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Estrogen Signaling and Endocrine Disruption Potential in the Embryonic Development of the Female Reproductive Tract of the American Alligator, Alligator mississippiensis

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Estrogen Signaling and Endocrine Disruption Potential in the Embryonic Development of the Female Reproductive Tract of the American Alligator (*Alligator mississippiensis)*

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A dissertation submitted to the faculty of the Medical University of South Carolina in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Graduate Studies.

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To my fathers, biological and scientific.
You created the opportunity;
my life's work is to prove worthy of it.

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This journey has been more rewarding, and much more difficult -- in more unexpected ways -- than any of my previous endeavors, and it would have never come to fruition without an amazing support network.

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AB – Alcian blue, a histochemical stain used to detect mucopolysaccharides, specifically acidic mucins

ACTB – beta-actin, a cytoskeletal protein used as an internal control gene/normalization factor in QPCR

AMH – anti-Müllerian hormone, a testis-produced protein that induces MD regression

AR – androgen receptor

DDE – dichlorodiphenyldichloroethylene, a metabolite of the pesticide DDT

DDT – dichlorodiphenyltrichloroethane, pesticide banned from use in 1972 in the U.S. due to documented detrimental effects on wildlife, particularly birds

DES – diethylstilbestrol, a synthetic estrogen; induced FRT abnormalities in women exposed *in utero* when it was prescribed as a miscarriage preventative from the 1940s-1970s

E_2 – estradiol-17β, endogenous estrogen in vertebrates

EB – estradiol benzoate, an estrogen ester

EEF1α – eukaryotic elongation factor 1α, a protein used as an internal control gene/normalization factor in QPCR

EGF – epidermal growth factor, morphogen that stimulates growth, proliferation and differentiation

EGFR – epidermal growth factor receptor

ER – estrogen receptor. There are two isoforms discussed in this work, ERα and ERβ. These abbreviations refer to the proteins.

ESR1 – the gene coding for ERα

ESR2 - the gene coding for ERβ

EW – egg weight

FPT – 30°C, the female producing temperature for American alligators during TSD

FRT – female reproductive tract

FSH – follicle stimulating hormone, gonadotropin that regulates ovarian folliculogenesis, used to induce reproductive system response in immature animals

GAM – gonad-adrenal-mesonephros complex, the embryonic alligator urogenital system

IGF1 – insulin-like growth factor 1, morphogen that stimulates growth, proliferation and differentiation

IGF1R – insulin-like growth factor 1 receptor

MD – Müllerian duct, the embryonic precursor of the female reproductive tract

MPT - 33°C, the male-producing temperature for American alligators during TSD

PAS – Periodic Acid Schiff's, a histochemical stain used to detect mucopolysaccharides, specifically glycosaminoglycans

PCNA – proliferating cell nuclear antigen, used as a marker of cellular proliferation

PFA – paraformaldehyde, a tissue fixative used for histological preparations

PGR – progesterone receptor

PPT – propyl pyrazole triol, specifically, 4,4',4''-(4-propyl-[1H]-pyrazole-1,3,5-triyl) trisphenol, a pharmaceutical ERα selective agonist

RPL8 – ribosomal protein L8, a 60S ribosomal subunit component used as an internal control gene/normalization factor in QPCR

RT-qPCR (or QPCR) – reverse transcription real-time quantitative polymerase chain reaction

SVL – snout-vent length, measured from the tip of the alligator snout to the center of the cloaca

SHR – steroid hormone receptor

TGF-β – transforming growth factor beta, a superfamily of cell regulatory proteins including the inhibins/activins that play an important role in ovarian folliculogenesis

TSD – temperature dependent sex determination

TSP – the thermosensitive period of temperature dependent sex determination

WAY – a pharmaceutical ERβ selective agonist, WAY 200070

WD – Wolffian duct, embryonic precursor of the male reproductive tract

ABSTRACT

BRENNA M DOHENY. Estrogen signaling and endocrine disruption potential in the embryonic development of the female reproductive tract of the American alligator, *Alligator mississippiensis*. (Under the direction of LOUIS J. GUILLETTE, JR. and SHERINE S.L. CHAN).

Perturbation of endocrine signaling during critical embryonic developmental windows has been implicated in many female reproductive system disorders. Reproductive tract anomalies in women exposed *in utero* to synthetic estrogen diethylstilbestrol, prescribed as a miscarriage preventative, provided some of the first evidence for this "embryonic origins of adult disease" paradigm and suggested a role for estrogen signaling in female reproductive tract (FRT) differentiation.

Developmental studies of FRT in other vertebrates have great value in furthering our understanding of embryonic origins of reproductive disorders. The alligator is a particularly intriguing model as an environmental sentinel species with temperature dependent sex determination, which allows for controlled manipulation of sexual differentiation in laboratory experiments. The signals involved in determining regionally specific cell fates in FRT of alligators and other crocodilians are not yet known, but multiple studies in their closest extant evolutionary relative, birds, underscore the role of estrogen signaling in this process.

Here I seek to characterize the role of estrogen signaling in the developing alligator FRT. I treated alligator embryos with estradiol-17β (E_2) and pharmaceutical agonists that are selective for their two estrogen receptor isoforms, ERα and ERβ. The ERα agonist, propyl pyrazole triol (PPT), induced significant enlargement of the developing FRT, compared with controls and E_2 and ERβ agonist treatments. Histological analysis revealed precocious glandulogenesis and connective tissue differentiation similar to mature FRT in these enlarged tissues. PPT treatment also altered expression of steroid hormone receptors and growth factor *IGF1*. Further experiments assessing this

CHAPTER 1:

General Introduction

1.1. Question formulation

At the heart of this dissertation is my underlying interest in the field of environmental endocrine disruption, which synergizes reproductive and developmental biology and endocrinology and has direct, far-reaching impacts on environmental health. The concept of environmental endocrine disruption arose when a growing scientific literature on reproductive impairments in both humans and wildlife led a trans-disciplinary group of experts to come together at a meeting in 1991 known as the Wingspread Work Session. Their consensus statement laid out the founding principles: that developmental exposure to hormonally active agents could disrupt normal developmental patterning at structural and functional levels that lead to disorders of the adult reproductive system (Colborn et al., 1992; Colborn et al., 1996).

The major human health evidence for this "developmental origins of adult disease" paradigm came in large part from the unanticipated adverse effects of a synthetic estrogen, diethylstilbestrol (DES), used as a miscarriage preventative from 1947-1971 (United States Centers for Disease Control and Prevention, 2011). This usage ceased when it was found that young women who were exposed *in utero* to DES developed an otherwise extremely rare cancer only found in much older women, vaginal clear cell adenocarcinoma (Herbst et al., 1971). Other structural disorders of the female reproductive tract (FRT) have been found in these "DES daughters," such as T-shaped uterus and vaginal adenosis, and these women face great difficulties in achieving and maintaining successful pregnancies (reviewed in Bern, 1992; Crain et al., 2008; Newbold, 2004).

DES exposure-induced FRT disorders are rooted in disruption of the normal differentiation of the embryonic Müllerian duct (MD) into specialized FRT regional

structures, the Fallopian tubes, uterus and upper vagina. Laboratory studies in mice have recapitulated the FRT disorders seen in human DES daughters, and have provided insight into the mechanisms underlying FRT disruptions due to embryonic DES exposure (reviewed in Newbold, 2004).

While laboratory models are essential for understanding the mechanisms underlying environmental endocrine disruption, wildlife models, exposed to real-world levels of environmental endocrine disrupting contaminants (EDCs) through various routes and in various mixtures, are equally critical for understanding potential health impacts. It was while reviewing studies on the effects of contaminants on wildlife in the Great Lakes region that Theo Colborn formulated the ideas that birthed the endocrine disruption hypothesis and led her to organize the Wingspread Work Session (Colborn et al., 1996).

The American alligator emerged as a particularly valuable wildlife model due to a discovery not unlike the story of DES exposure in humans. A severe population decline in alligators living in Lake Apopka, FL, following a major spill of the pesticide dichlorodiphenyltrichloroethane (DDT) in 1980, led to the recruitment of Louis J. Guillette, Jr., by the Florida Fish and Wildlife Conservation Commission (then Florida Game and Freshwater Fish Commission) to investigate this anomalous population, as well as overarching questions of alligator reproductive biology.

By comparing alligators in Lake Apopka with alligator populations in more pristine Florida lakes, Dr. Guillette's research group and collaborators established that Apopka alligators had higher serum, tissue and egg yolk levels of many contaminants, most notably DDT metabolite *p,p'*-dichlorodiphenyldichloroethylene (DDE) (reviewed in Woodward et al., 2011). Experiments involving field collection of eggs and controlled incubation in the laboratory showed that Apopka alligators developed altered gonadal and external genitalia morphology compared with alligators from a reference site (reviewed in Guillette et al., 2000; Guillette and Moore, 2006; Milnes and Guillette, 2008). These

results strongly suggested that developmental exposure to EDCs, transferred from mother to embryo via yolk, were the basis for the adverse outcomes seen in Apopka alligators: the decreased viability and hatching success of eggs as well as the reproductive system anomalies (Hamlin and Guillette, 2011).

This long-term case study coupled with the unique attributes of alligators have led to more than three decades of research in our laboratory utilizing the American alligator as a sentinel for studying environmental influences on reproductive health. Alligators are long-lived, and have a very high site affinity, which offers the opportunity to correlate reproductive health anomalies with contaminant exposure. Their semi-aquatic lifestyle and position as top predators also make alligators particularly susceptible to exposure to EDCs, many of which biomagnify across trophic levels and bioaccumulate in tissues (Milnes and Guillette, 2008). What really sets alligators apart as a model species, however, is that sex determination in the alligator is temperature dependent, rather than genetic (Ferguson and Joanen, 1982; Ferguson, 1985). Incubation of alligator eggs at 30°C during a critical window known as the thermosensitive period (TSP) produces all females, while incubation at 33°C during the TSP produces all males (Ferguson and Joanen, 1982; Lang and Andrews, 1994). This makes the alligator an ideal experimental model for research involving sexual differentiation, and the effects of environmental factors such as EDCs on this process, as the sex of developing embryos can be controlled and manipulated in the lab via incubation temperature.

In efforts to better understand alligator reproductive health, adult alligator FRT structure and function and adult reproductive endocrine physiology have been studied by our research group. The development of the alligator FRT has been a neglected area of inquiry, however. Considering the significance of FRT development in the field of endocrine disruption research thanks to the pivotal role the DES story played in its formulation, the underlying question for my dissertation research arose quite organically: ***Is the developing female reproductive tract of the American alligator a potential target for endocrine disruption?***

1.2. Background

1.2.1. Female reproductive tract development in mammals

In the mammalian system, with XX/XY chromosomal genetic sex determination, the FRT pathway has long been considered to be the "default" state. During early development, the embryo develops a bipotential gonad and both male (Wolffian duct) and female (Müllerian duct, MD) reproductive tract primordia. The transcription factor *Sry* on the male Y chromosome is necessary to induce differentiation of the early bipotential gonad to testis, which produces anti-Müllerian hormone (AMH) and testosterone to induce MD degeneration and Wolffian duct (WD) promotion. In the absence of these male-promoting factors, the MD differentiates into the FRT, which encompasses the fallopian tubes, uterus, cervix and upper vagina. (reviewed in Gilbert, 2000; Kobayashi and Behringer, 2003; vom Saal et al., 1992; Yin and Ma, 2005)

Despite this "default" nature of mammalian FRT development, studies have shown that there are many important genetic factors involved in promoting regional differentiation of the MD (reviewed in Kobayashi and Behringer, 2003). Of interest when considering the potential role of estrogen signaling in FRT development is the regional expression of Homeobox (Hox) genes, the master regulators of axial patterning during embryogenesis. The chromosomal position of these genes from 3' to 5' corresponds with their anteroposterior expression along the body axis of the developing embryo (Krumlauf, 1994). In the developing mammalian FRT, regional expression of the Hoxa 5' genes correlates directly with regional differentiation. Hoxa9 is expressed in the developing oviduct, Hoxa10 in the uterus, Hoxa11 in the cervix and Hoxa13 in the upper vagina (Taylor et al., 1997). This regional expression of Hox genes is also present in the adult FRT and related to fertility and implantation success. Targeted disruption of either Hoxa10 (Satokata et al., 1995) or Hoxa11 (Hsieh-Li et al., 1995) results in female infertility. Experimental evidence suggests estrogen signaling is involved in regulation of

regional expression of Hoxa 5' genes in the developing FRT. Developmental exposure to DES in mice results in a posterior shift of Hoxa9, Hoxa10 and Hoxa11 gene expression, accompanied by reproductive tract abnormalities indicative of a posterior shift of specialized FRT structures (Block et al., 2000; Ma et al., 1998).

Whether estrogen signaling plays a role in normal mammalian FRT development is uncertain. Estrogens have been shown to protect the MD from degeneration induced by testosterone and AMH (reviewed in vom Saal et al., 1992). In estrogen receptor knockout mouse models, the MD differentiates into the normal FRT regional structures, but the uterus is much smaller in size and unresponsive to estrogen (reviewed in Emmen and Korach, 2003). It is hypothesized that the mammalian MD undergoes an estrogen-independent "organogenetic" differentiation in early development that determines regional structures, and an estrogen driven "functional" differentiation at reproductive maturity during estrous cycles to allow for pregnancy (Kurita, 2011).

1.2.2. Female reproductive tract development in birds

Avian sex determination is chromosomal, with a ZZ/ZW system, but in contrast to mammals, the female is the heterogametic sex. The actual genetic factors involved in gonadal sex differentiation in birds were unknown until quite recently. A Z-linked gene, *DMRT1*, is involved in testis determination in a dosage-dependent fashion, in that two copies, found in ZZ males, are necessary (Smith et al., 2009). In a ZW female, the left gonad differentiates into an ovary, which produces estrogens that counter the effects of AMH, leading to oviduct development (Hamilton, 1961; reviewed in Hermansson, 2007). In most bird species, aside from the brown kiwi and some falconiformes, only the left ovary and oviduct develop (Johnson, 1986). Both male and female gonads produce AMH, but only the left gonad in females produces estrogen to counter its effects, and the left MD has a higher abundance of estrogen receptors. This protects the left MD and promotes oviductal growth and differentiation, while the right gonad atrophies and

the right MD degenerates (Hutson et al., 1985; Hutson et al., 1983; MacLaughlin et al., 1983). It is thought that this asymmetrical female reproductive system evolved as one of many adaptations that make birds capable of flight (Witschi, 1935).

Estrogen appears to be a necessary factor in normal avian FRT differentiation. Female embryos have higher plasma estradiol than males (Woods and Brazzill, 1981), and administration of estrogen to male embryos results in MD retention (Willier et al., 1937). *In ovo* treatment with an aromatase inhibitor while the gonads are in a bipotential state results in sex reversal of genetically female chickens, with full testicular development and absence of both oviducts (Elbrecht and Smith, 1992).

The developing avian FRT is particularly sensitive to exogenous estrogenic input, and in fact there is a long history of research utilizing the developing avian oviduct as a test system for studying estrogen action (reviewed in Dougherty and Sanders, 2005). Studies in the 1930s and 1940s documented left oviductal hypertrophy and retention of the right oviduct when immature chickens were dosed with exogenous estrogens (Munro and Kosin, 1943). Further studies have shown that *in ovo* estrogenic stimulation induces cytodifferentiation and glandular morphogenesis in the avian oviduct (Andrews and Teng, 1979; Berg et al., 2001; Kohler et al., 1969; Oka and Schimke, 1969b).

Similarly to the DES story in humans, wild birds have suffered unintended FRT disruption thanks to the widespread use of the insecticide DDT for mosquito control from the time of WWII until its ban in 1972. Exposure to DDT and its persistent metabolite *p,p'*-DDE was directly correlated with eggshell thinning and other reproductive anomalies, which led to serious declines in exposed populations (reviewed in Cooke, 1973). Adult exposure to DDT was thought to result in impaired shell formation in the oviduct via inhibition of carbonic anhydrase (Bitman et al., 1969), but more recent studies have supported the endocrine disruption paradigm of developmental exposure leading to adult disorders. *In ovo* exposure to DDT resulted in reproductive dysfunctions in adult chickens, including production of eggs with significantly thinner, or even absent,

shells (Hermansson, 2007; Holm et al., 2006). Studies in Japanese quail have also shown disruptions of important oviductal structures and reduced expression of calcium regulating factors due to *in ovo* exposure to DDT (Kamata et al., 2009).

1.2.3. The alligator female reproductive tract

The adult alligator FRT is a highly regionalized tissue, in which seven morphologically and functionally distinct regions have been described: the anterior infundibulum, posterior infundibulum, tube, uterotubular junction, anterior uterus, posterior uterus, and vagina (Fig. 1.1). As members of the Archosaurian evolutionary lineage, encompassing birds, crocodilians and extinct dinosaurs, female alligators have a reproductive tract that shares more similarities with birds than other reptiles (Palmer and Guillette, 1992). In non-Archosaurian amniotes, the uterine region produces both the fibrous proteins that form the eggshell membrane and the outer calcareous layer of the eggshell, in a sequential process (Palmer, 1990). In Archosaurs, there are two spatially distinct regions, complete with distinct glandular structures that produce these different secretory products, identified here in the alligator as the anterior and posterior uterus, but also referred to as the "fiber region" and "calcium region" (Palmer, 1990; Palmer and Guillette, 1992). Thus Archosaurian eggs can be packaged in an "assembly line" fashion, an evolutionary adaptation that has been seen as an innovation allowing for flight in birds (Guillette, 1991). The major differences between crocodilians and their winged counterparts are that crocodilians retain paired reproductive structures, and process an entire clutch of eggs through their oviducts at once, while birds only retain the left reproductive organs and process their eggs through their oviducts one at a time (Palmer, 1990).

As described by Palmer and Guillette (1991), three regions of the alligator FRT are of particular functional importance: the tube, the anterior uterus and the posterior uterus. On a structural and functional level, the tubal region has a tall columnar

epithelium of ciliated and microvillous secretory cells and an extensive network of branched acinar glands that secrete glycosaminoglycans. The tube is the most biosynthetically active region of the reproductively active alligator FRT, and it produces albumen and ovotransferrin proteins (Buhi et al., 1999). The anterior uterus secretes protein fibers that form the eggshell membranes (Buhi et al., 1999) from branched tubular glands with numerous visible eosinophilic secretory granules. The posterior uterus is the site of the shell gland, involved in producing eggshells, and the branched tubular glands have fewer secretory granules than the anterior uterus, and the luminal epithelium is lower (Palmer and Guillette, 1992).

When reproductively active, the alligator FRT undergoes dramatic changes in size. A non-reproductive female two meters in total body length reportedly has oviducts of 45 cm in length, which can grow to be 1.5-2.5 m in length under stimulation by endogenous estrogen during the reproductive cycle (Guillette et al., 1997). This spike in estrogen signaling also induces extensive glandular development to enable the FRT to produce all of the secretory products necessary for proper egg packaging (Palmer, 1990).

1.2.4. Estrogens and alligator female reproductive tract development

Considering the response of the adult alligator FRT to estrogen during reproductive cyclicity, and the shared Archosaurian lineage between birds and alligators, it is probable that estrogens play a role in the developing FRT of the alligator similar to that in the developing avian FRT. Early studies on alligator FRT development support this hypothesis. In the 1930s, Thomas Forbes studied the role of steroid hormones in alligator reproductive system development by treating 15-month-old juvenile female alligators with large daily injections of estradiol benzoate (EB) over an 80 day period, resulting in massive oviductal hypertrophy (Forbes, 1938a; Forbes, 1938b). Over 50 years later, in studies on alligator MD regression, Austin (1989) noted that neonatal exposure to estrogen via implanted pellets also induced oviductal hypertrophy.

Experimental treatment with hormones *in ovo* has also been shown to influence alligator FRT development. In their in-depth investigation on the TSP of sex determination in alligators, Lang and Andrews (1994) showed that dosing embryonic alligators with estradiol-17β (E_2) prior to incubation at the male producing temperature (MPT) during the TSP resulted in 100% gonadal sex reversal (i.e. development of ovaries rather than testes), and "some" oviductal hypertrophy relative to untreated animals incubated at the female producing temperature (FPT). Hypertrophied MDs were also reported following *in ovo* treatment with norethindrone, an aromatase inhibitor and synthetic progestin (Austin, 1991).

None of these experiments documented differentiation of the MD into mature FRT structures on either a morphological or histological level, however. This underscores a fundamental lack of knowledge on alligator FRT development, as it is unknown when the alligator MD normally undergoes regional differentiation. In his estrogen treatment study, Forbes (1938) noted that in juvenile alligators, over 1.5 years old by the end of the dosing regime, the oviducts of the control animals were completely undifferentiated, displaying the same simple columnar luminal epithelium and undifferentiated mesenchymal stroma observed in embryos and neonates (Forbes, 1940). While the oviducts were massively hypertrophied in treated females, they also lacked adult FRT characteristics; Forbes noted ciliation and increased height of the luminal epithelial cells and some smooth muscle differentiation in outer stroma, but no glandular development or distinctive regional characteristics (Forbes, 1938a). This matches what has been heretofore seen by our laboratory members in our alligator research – no differentiation of the MD or oviduct in alligators we have sampled at embryonic, neonatal, or juvenile stages up to 1.5 years of age. In mammals, the MD differentiates into the FRT structures before birth (humans) or not long thereafter, with full differentiation of uterine glands completed during puberty (Yin and Ma, 2005). The age at which alligator MD differentiates into regionalized FRT, and the signals involved in this process, are thus far unknown.

1.2.5. Estrogen receptor isoforms and the induction of an unexpected alligator Müllerian duct phenotype

In 2010 we conducted an experiment to investigate the effects of *in ovo* treatment with estrogen receptor (ER) isoform specific agonists on alligator sex determination. Exposure to exogenous estrogen can induce ovarian development in alligator embryos incubated at the MPT (Bull et al., 1988; Kohno and Guillette, 2013) and exposure to estrogenic contaminants can alter sex ratios at an intermediate temperature (Crain et al., 1997; Milnes et al., 2005), suggesting that an estrogenic signal prior to TSP overrides the male-determining temperature signal. To better understand the molecular mechanism involved in this process, we treated embryos with pharmaceutical agents with specific affinities for the ER isoforms, ERα and ERβ.

There are at least two isoforms of the ER that have been identified in all vertebrate classes, which have high sequence identity in their DNA binding domains but less so in their ligand binding domains (Katzenellenbogen et al., 2000b; Thornton, 2001). These structural differences are borne out in differential affinities between the two isoforms for exogenous ligands (Li et al., 2012).

Studies in the red-eared slider turtle, another reptile with TSD, showed differential expression patterns of ERα and ERβ in the developing gonad, and differences in ovarian localization in response to temperature or stimulation with E_2 (Ramsey and Crews, 2009). This led us to consider the potential roles of these ER isoforms in TSD in the alligator. Via receptor transactivation assays, we identified pharmaceutical agonists selective for the alligator ER isoforms. *In ovo* treatment with the ERα selective agonist propyl pyrazole triol (PPT) induced ovarian differentiation at the MPT, while treatment with the ERβ selective agonist WAY 200070 (WAY) did not (Kohno et al., 2015).

In addition to its potential role in gonadal differentiation, the interplay of signaling between ERα and ERβ appears to be an important regulatory mechanism in the FRT.

Mammalian studies suggest that estrogen-mediated cellular proliferation in the uterus occurs through ERα, as an ERα knockout mouse model displays decreased uterine growth compared to wild type (Hewitt et al., 2010). Further evidence in multiple estrogen signaling pathways suggest an antagonistic relationship between the ER isoforms, with ERβ antagonistically regulating ERα activity (reviewed in Matthews and Gustafsson, 2003). Additionally, ER knockout mouse studies as well as studies using ER-isoform specific agonists in mice have shown that the FRT anomalies induced by perinatal DES exposure are mediated through ERα (Couse and Korach, 2004; Nakamura et al., 2008).

In our experiment, *in ovo* treatment with the ERα selective agonist PPT unexpectedly induced extreme MD enlargement. Neither treatment with E_2 nor with the ERβ selective agonist WAY at comparable doses had this effect (Kohno et al., 2015). Altered MD morphology at this level had never previously been observed in our studies. Even when using substantially higher doses of *in ovo* administered E_2, the only observed oviductal effect was increased luminal epithelial cell height (Crain et al., 1999).

This finding raised many questions around the role of estrogen signaling in alligator FRT development. I was intrigued that such a dramatic morphological change occurred due to selective agonism of one ER isoform, when many other experiments with endogenous and synthetic estrogens and estrogenic chemicals produced no observable MD effects. This PPT-induced MD phenotype initiated my dissertation project, as it provided a model for producing investigable alterations in developing alligator FRT rooted in altered endocrine signaling.

1.3. Research goal and questions for investigation

The goal of this dissertation research is **to characterize the role of estrogen signaling in the development of the female reproductive tract of the alligator, in order to provide a better understanding of how exposure to EDCs during critical developmental windows can disrupt this system.**

The fortuitous discovery of the PPT-induced MD phenotype provided a jumping-off point for further investigation. Three main questions stemming from this discovery are addressed in the following research chapters:

1. What are the histological and molecular characteristics of the PPT-induced MD phenotype, and can they be utilized to inform further exploration into the role of estrogen signaling in alligator FRT development?

2. Why does only treatment with ERα selective agonist PPT, and not treatment with E_2 or ERβ selective agonist WAY, induce these MD characteristics?

3. Do alligators from a background of contaminant exposure exhibit a different MD response to PPT treatment than alligators from a reference site?

These questions are addressed in the following experiments based on utilizing the established model of *in ovo* treatment with E_2 and selective pharmaceutical agonists for ERα and ERβ, and comparing their effects on alligator MD via histological assessment and analysis of expression of key genes of interest.

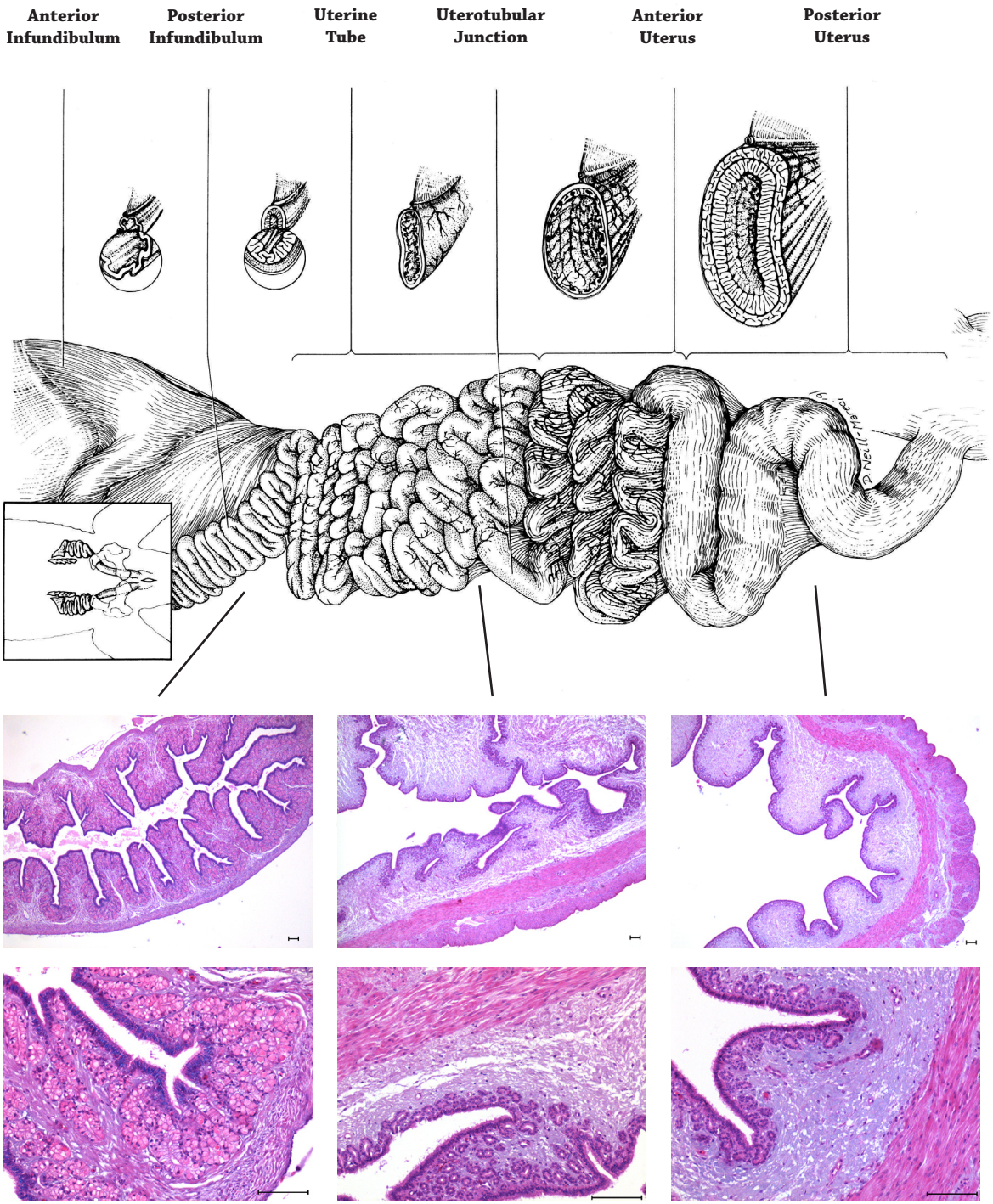


Fig. 1.1 *The adult alligator female reproductive tract is highly regionalized.* Schematic drawing (modified from Guillette, 1992) of the adult alligator FRT external and cross sectional morphology. Below are representative cross sectional photomicrographs of the three indicated regions, tube, anterior uterus, and posterior uterus, at 4x (above) and 20x (below) magnifications. Sections stained with H&E. Scale bars = 100 μm.

CHAPTER 2:

In ovo treatment with an estrogen receptor alpha selective agonist causes precocious development of the female reproductive tract of the American alligator (*Alligator mississippiensis*)

2.1. Introduction

The American alligator has featured in multiple studies of environmental sex determination and the effects of environmental endocrine disruptors that have provided valuable insights into the lasting effects of early developmental exposures (Kohno and Guillette, 2013; Milnes and Guillette, 2008). Sex determination in the alligator is temperature dependent, rather than genetic (Ferguson, 1985), which makes it an interesting experimental model for research involving sexual differentiation, as the sex of developing embryos can be manipulated in the laboratory via incubation temperature. Moreover, administration of estrogens or ERα agonists to embryos incubated at the MPT induces ovarian development (Bull et al., 1988; Kohno et al., 2015; Kohno and Guillette, 2013). While multiple studies have investigated the effect of estrogens on ovarian differentiation in reptile species exhibiting TSD, the hormonal influences on the differentiation of the MD into the mature oviduct (the female reproductive tract structures including infundibulum, tube and uterus) remains largely unknown. In the alligator, this differentiation of the oviduct occurs sometime after hatching and before reproductive maturity, but the actual time point remains undocumented. For the purposes of this study, the female reproductive tract will be designated “Müllerian duct” in the embryo and “oviduct” after hatch out.

In birds, which share the Archosaurian evolutionary lineage with crocodilians, differentiation of the MD is particularly sensitive to estrogenic input. Early studies documented left oviductal hypertrophy and retention of the right oviduct when immature chickens were dosed with exogenous estrogens (Munro and Kosin, 1943). In the current

understanding of the development of the avian oviduct, genetic factors lead to ovarian differentiation of the left gonad, which produces estrogens that counter the effects of AMH, leading to oviduct development (reviewed in Hermansson, 2007). Estrogens are also involved in differentiation of the avian MD. *In ovo* estrogenic stimulation induces proliferation of the luminal epithelium and cytodifferentiation and glandular morphogenesis in the avian oviduct (Andrews and Teng, 1979; Berg et al., 2001).

Several studies suggest that the alligator MD is similarly labile to the influence of estrogen signaling. In some studies of sex determination in alligators, E_2 treatment of eggs incubated at MPT resulted in 100% ovarian development and "some" oviductal hypertrophy relative to untreated animals incubated at the FPT (Bull et al., 1988; Lang and Andrews, 1994). Treatment of eggs incubated at FPT with a synthetic progesterone, norethindrone, also led to hypertrophied MDs (Austin, 1991). Postnatal estrogenic treatment of hatchling and juvenile female alligators up to 1.5 years of age has also been shown to induce oviductal hypertrophy (Austin, 1989; Forbes, 1938a; Forbes, 1938b). These studies support the hypothesis that the developing alligator FRT is receptive to estrogen stimulation and is a potential target of disruption by environmental EDCs.

The ER is highly promiscuous, having an affinity for a wide variety of compounds including endogenous and synthetic estrogens, plant hormones, and EDCs such as bisphenol A and agricultural pesticides and their metabolites (Katzenellenbogen, 1995). There are two isoforms of ER that have been identified in amniotes, ERα and ERβ, which have high sequence identity in their DNA binding domains but less so in their ligand binding domains (Katzenellenbogen et al., 2000a; Katzenellenbogen et al., 2000b). Mammalian studies of MD differentiation indicate an interplay of estrogen signaling between the two receptor subtypes, with ERα mediating estrogen-induced cellular proliferation. ERα knockout mice display decreased uterine growth linked to dysregulation of insulin-like growth factor-1 (IGF1) (Hewitt et al., 2010). Similarly, ER knockout mice as well as studies utilizing ER-isoform selective agonists in mice have

shown that reproductive tract anomalies induced by exogenous estrogen exposure act through ERα (Couse and Korach, 2004; Nakamura et al., 2008).

Both ERs from the American alligator have been cloned and sequenced (Katsu et al., 2004; Katsu et al., 2010). As with other vertebrates, alligator ERs show high specificity for endogenous estrogens in their transactivation (Katsu et al., 2010; Kohno et al., 2015). Further studies have shown that both receptor subtypes, as well as the androgen receptor (AR) and progesterone receptor (PGR), are expressed in oviduct of the juvenile alligator (Moore et al., 2012).

In a previous study, an ERα selective agonist, PPT, induced an unusual MD phenotype in alligator embryos incubated at the MPT (Kohno et al., 2015). This was of interest for further investigation, as the embryonic development of the alligator MD is not well understood, and no previous studies have reported the particular MD phenotype we observed.

Here we investigated the effects of *in ovo* stimulation of the ER subtypes with selective agonists in embryos incubated at the FPT to assess the effects of receptor subtype-specific estrogen signaling on alligator oviductal development. We also examined the expression of MD genes involved in steroid hormone signaling and proliferation induced by estrogenic treatment in the developing female alligator.

2.2. Materials and methods

2.2.1. Egg collection, incubation and treatment

All experiments in this study involving alligators were carried out under the protocol approved by the Institutional Animal Care and Use Committee at the Medical University of South Carolina, and all fieldwork was conducted under permits from the Florida Fish and Wildlife Conservation Commission and the U.S. Fish and Wildlife Service.

Alligator eggs were collected from Lake Woodruff National Wildlife Refuge (De Leon Springs, FL, USA) under Florida Fish and Wildlife Commission permits on 6/15/2012 and 6/21/2012. Upon transportation to our facilities at the South Carolina Department of Natural Resources Marine Resources Research Institute (Charleston, SC, USA), eggs were weighed, candled to ascertain viability and placed in nest boxes of moist sphagnum moss and incubated at 30°C (FPT) in enclosed incubators. An embryo from each clutch was sampled and staged based on criteria from Ferguson (1985).

Once the embryos had developed to stage 19, as predicted by equation (Kohno and Guillette, 2013), they were randomly assigned to treatment groups: 0.5 μL/g egg weight (EW) absolute ethanol; 0.005, 0.05 and 0.5 μg/g EW E_2 (Sigma-Aldrich, St. Louis, MO, USA); 0.05, 0.5, and 5 μg/g EW PPT (Tocris Bioscience, Bristol, UK); and 0.05, 0.5 and 5 μg/g EW WAY 200070 (Tocris). These doses were based on the doses of PPT and E_2 that previously induced ovarian development in embryos at MPT (Kohno et al., 2015). All compounds were dissolved in absolute ethanol (95% ethanol incubated for 24 hours with molecular sieves). Eggs were weighed and treated by painting the eggshell surface with the appropriate dose of treatment solution (Crews and Wibbels, 1993). Embryos were incubated at FPT for the duration of the experiment.

Embryos were sacrificed at stage 27, which is the stage prior to hatching. At time of collection, eggs were re-weighed, and embryos were weighed and sacrificed. Left and right gonad-adrenal-mesonephros (GAM) complexes, with attached MDs, were collected under a dissecting microscope, the right preserved in RNA*later*® (Life Technologies, Grand Island, NY, USA) and the left in 4% paraformaldehyde (PFA) fixative. The fixed tissues were incubated overnight on a shaker at 4°C. The RNA*later*® -preserved tissues were then stored at -20°C. The PFA-fixed tissues were progressively dehydrated in methanol/PBST over a 24-hour period and stored in 100% methanol at -20°C.

2.2.2. Histology

GAM tissues with attached MDs were examined for gross morphological characteristics under a dissecting microscope. Representative samples were photographed using a PixeLINK digital camera and the PixeLINK Capture OEM software (Ottowa, ON, Canada).

The PFA-fixed tissues were further dehydrated through a series of isopropanol and xylene and paraffin infiltrated using a Leica ASP300 autoprocessor (Wetzlar, Germany), and embedded into paraffin using a Leica EG1150C embedder. Tissues were serially cross-sectioned at 7 μm using a Leica RM2255 microtome, and stained with hemotoxylin and eosin.

Full serial cross sections were examined for a subset of the samples in each treatment group ($n \geq 5$) to determine regions of interest for direct comparison. The anterior-posterior axis of the GAM was utilized as a metric for assessing regionalization of the MD. Representative digital photomicrographs were taken with a ProgRes Speed XT core 5 camera (Jenoptik, Jena, Germany) of a region near the anterior end of the GAM, and at a region just posterior of the adrenal gland, around the midpoint of the GAM complex. Using the IMT iSolution Lite software (Martin Microscope Company, Easley, SC, USA), luminal epithelial cell height was measured at four points in three separate sections at each of the regions examined ($n = 5$ samples from each treatment group).

2.2.3 RNA isolation, cDNA synthesis and quantitative PCR

RNA*later*®-preserved MD tissues were manually separated from GAM complexes under a dissecting microscope and stored at -20°C until RNA extraction. Whole MDs were homogenized for RNA extraction. Methods for RNA isolation, cDNA synthesis and qPCR have been previously described (Kohno et al., 2015). RNA was isolated from tissues using the acid phenol-guanidinium thiocyanate-chloroform

extraction method followed by further purification using the SV Total RNA Isolation System with DNase-I treatment (Promega, Fitchburg, WI, USA). RNA concentration was measured using a Nanodrop Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and quality was assessed via denaturing agarose gel electrophoresis. Prior to cDNA synthesis, RNA was diluted to matching concentrations and used as a template for quantitative real-time PCR to check for genomic DNA contamination. RNA was reverse transcribed using the iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA) per manufacturer's protocol with 15 µL RNA in 20 µL total reaction volume, and the resulting cDNA was diluted 1:5 in nuclease-free water.

QPCR was performed using a CFX96 real-time system (Bio-Rad). Reactions were performed in triplicate using 0.01 U/µl AmpliTaq Gold (Applied Biosystems, Carlsbad, CA, USA) and an in-house SYBR green reaction mixture consisting of 20mM Tris-HCL (pH 7.75), 50mM KCl, 3mM $MgCl_2$, 4% DMSO, 0.5x SYBR Green I (Thermo Fisher Scientific, Grand Island, NY, USA), 0.5% glycerol, 0.5% Tween 20, 0.2mM deoxynucleotide mix (Thermo Fisher Scientific), 0.2 µM primer mix (Eurofins Genomics, Huntsville, AL, USA), and 1/25 volume diluted cDNA.

The thermal cycler program consisted of an initial denaturation step (95°C, 5 min) followed by 45 cycles of denaturation (95°C, 15 s) and annealing and elongation at an optimized temperature (45 s). After the final cycle, a dissociation curve was generated for each reaction to assess the reaction specificity via an incremental heating step (+0.5°C/5 s).

Primer sequences, product size and optimal annealing temperatures are listed in Table 1. Prior to analysis of unknown samples, the specificity of each primer pair was confirmed by product sequencing. Plasmid DNA standard curves of known copy number were generated with these sequenced products. Each 96-well plate of unknown samples included a standard curve, and all samples, including those of the non-template control and standard curve, were run in triplicate. Mean expression values of mRNA for each target gene were normalized to that of ribosomal protein L8 (*RPL8*). Due to low mRNA

levels for *ESR2*, this gene was re-run using full concentration cDNA (not 1:5 diluted) and normalized to the values for *RPL8* with non-diluted cDNA.

2.2.4. Statistical analysis

The effect of treatment on mean luminal epithelial cell height at both regions histologically examined was analyzed via one-way analysis of variance (ANOVA) followed by a Tukey's multiple comparison post-hoc test.

QPCR data were collected and analyzed using CFX Manager version 3.0 software (Bio-Rad). After determining that expression of *RPL8* was not significantly affected by treatment via one-way ANOVA, expression values of all other genes of interest were normalized to *RPL8*. Arcsine-transformed data was used to conduct one-way ANOVA to calculate the effect of treatment on expression of genes of interest, and outlying values were removed via ROUT method with a maximum false discovery rate set at 1%. Significance was set at $p < 0.05$, and a Tukey's multiple comparison post-hoc test was used to detect differences among treatment groups.

All statistical analysis was performed using both JMP12 (SAS Institute, Inc., Cary, NC, USA) and Graphpad Prism 6 (Graphpad Software, Inc., LaJolla, CA, USA) software.

2.3. Results

2.3.1. *Gross morphology of the embryonic Müllerian duct is altered by PPT treatment*

The reproductive organs of the embryonic female alligator at stage 27 include the paired GAM and the MDs, simple tubes that run along the distal portion of the GAM, connected to the GAM by the mesosalpinx, and terminate in the cloaca. Treatment with 5 μg/g EW PPT at FPT produced enlarged MDs, thickened pubis symphysis, and enlarged cloaca compared to controls. As shown in Figure 2.1, compared with the vehicle control samples, the MDs of the high dose (5 μg/g EW) PPT-treated tissues were much larger

in diameter, dwarfing the GAM complex with their size and length, extending farther anteriorly into the abdominal cavity. While control MDs were relatively straight, simple tubes that ran alongside the GAM, 5 μg/g EW PPT-treated MDs were so expanded in size and length that they were tortuous rather than lying flush with the GAM.

Treatment with 0.5 μg/g EW PPT also induced MD enlargement, though it was not as extreme as the higher dose of 5 μg/g EW PPT, and the low dose PPT treatment (0.05 μg/g EW) did not induce any observable MD alterations (Fig. 2.1). None of the samples treated at any of the doses of the ERβ agonist, WAY, or the doses of E_2, showed significant gross morphological differences from the control (Fig. 2.1).

2.3.2. *PPT treatment induced glandular development in the M*üllerian duct

At stage 27 the embryonic GAM and MD tissues were connected by a mesosalpinx and were most easily processed for histology as a complex. These GAM-MD complexes were serially cross-sectioned and two regions along the anterior-posterior axis were selected for close histological examination (Fig. 2.2). At this stage, the control MD was structurally simple, with a central lumen with a simple columnar luminal epithelium, surrounded by a fibroblastic mesenchymal stroma (Fig. 2.3). There was a short region around the midpoint of the GAM complex, just posterior to the terminus of the adrenal gland, in which the epithelial cells lining the oviductal lumen displayed an increased height (Figs. 2.3B and D). This regional characteristic was displayed in all samples in all treatment groups examined in this study, so comparative observations were taken at two regions along the GAM-MD complex, as indicated in Fig. 2.2.

The histological cross sections of the medium (0.5 μg/g EW) and high dose (5 μg/g EW) PPT-treated MDs were strikingly different from all other treatment groups, as was expected due to the obvious gross morphological differences (Figs. 2.4 and 2.5). In eight out of 11 medium dose (0.5 μg/g EW) PPT-treated tissues, the MD was greatly enlarged in width compared to the controls, with hypertrophied stromal cells (Fig. 2.4).

However, no cellular differentiation was observed in the stroma or the epithelium in tissues treated at this dose.

More striking characteristics were observed in the high dose (5 μg/g EW) PPT treatment group, which displayed not only overall MD enlargement, but also cellular differentiation of the stroma (Fig. 2.5). Multiple glandular structures budded out from the luminal epithelium, and there was secretory material present in the lumen (Figs. 2.5A and C). Proceeding posteriorly along the oviduct, these glandular structures were less numerous and there was a greater proportion of connective tissue in the stroma (Figs. 2.5B and D). None of the other treatment groups, including the 0.5 μg/g EW PPT treatment group, developed any of these differentiated structures.

*2.3.3. PPT treatment induces increased luminal epithelial cell height in the embryonic alligator M*üllerian *duct.*

The MD luminal epithelium was also altered by PPT treatment. The epithelium of the MD at stage 27 in the controls was simple columnar (Figs. 2.3A and C), with a region of greater proliferation and increased cell height near the middle of the GAM complex (Figs. 2.3B and D). The epithelial cell heights at both the anterior and middle region of the 5 μg/g EW PPT-treated MDs were significantly higher than all other treatment groups (p <0.0001, Fig. 6). The high dose (5 μg/g EW) PPT-treated luminal epithelial cells are also ciliated, a characteristic that none of the other treatment groups displayed (Figs. 2.5C and D).

2.3.4. PPT treatment alters gene expression in the embryonic Müllerian duct

ERα mRNA expression in MD was reduced nearly threefold by 5 μg/g EW PPT treatment (Fig. 2.7). *ERβ* and *PGR* mRNA levels were very low in embryonic MD of controls, but PPT treatment significantly increased them at both the medium (0.5 μg/g EW) and high dose (5 μg/g EW) levels (Fig. 2.7). *PGR* mRNA was 12-fold higher at the

medium dose and 25-fold higher at the high dose of PPT-treatment than controls (Fig. 2.7). *AR* expression in MD was increased threefold by 5 μg/g EW PPT treatment (Fig. 2.7). *IGF1* expression in MD was increased five-fold at both 0.5 μg/g EW and 5 μg/g EW PPT doses (Fig. 2.7).

Expression of IGF1 receptor (*IGFR*), epidermal growth factor (*EGF*), EGF receptor (*EGFR*) and proliferating cell nuclear antigen (*PCNA*) were not significantly altered by the treatments (Fig. 2.8).

2.4. Discussion

The MD phenotype induced by *in ovo* treatment with 5 μg/g EW PPT was substantially different from controls and all other treatments in this experiment. The histological characteristics of these high-dose PPT-treated MD are more similar in appearance to the vitellogenic adult alligator oviduct than to what has been previously observed and documented in normal stage 27 alligators incubated at FPT. The mature alligator oviduct is differentiated into highly specific regions with distinct characteristics and functions. Three regions are of particular importance: the tube, the anterior uterus and the posterior uterus. The tubal region has a tall columnar epithelium of ciliated and microvillous secretory cells and an extensive network of branched acinar glands that secrete glycosaminoglycans (Bagwill et al., 2009; Palmer and Guillette, 1992). The anterior uterus secretes protein fibers from branched tubular glands with numerous visible eosinophilic secretory granules, while the posterior uterus is the site of the shell gland, and the branched tubular glands have fewer secretory granules than the anterior uterus. Progressing posteriorly along the oviduct, stromal glands become less pronounced and there is a greater area of connective tissue, surrounded by layers of smooth muscle. (Bagwill et al., 2009; Palmer and Guillette, 1992).

While not directly congruent with the adult oviduct, the MDs of the embryonic alligators treated with 5 μg/g EW of PPT in this study display similar regional

characteristics. A higher proportion of glands fill the stroma at the anterior end, and these glands become less prominent moving posteriorly along the tissue. Connective tissue becomes more obvious in the posterior regions of the MD, and the outer portion appears to be differentiating into smooth muscle.

While previous studies have shown oviductal hypertrophy following treatment with estrogens, no studies document glandular differentiation in the immature alligator oviduct or embryonic MD. Forbes (1938b) noted smooth muscle differentiation in the outer stroma in the hypertrophied oviducts of 15-month-old alligators injected 3 or more times a week for 80 days with large doses of EB. This smooth muscular differentiation increased posteriorly, similarly to the regionalized characteristics in adult oviducts (Bagwill et al., 2009; Palmer and Guillette, 1992) and in the 5 μg/g EW PPT-treated MDs in this study. There is only one study in the literature that documents histological characteristics of hypertrophied oviducts induced by *in ovo* treatment, in this case with the synthetic progestin norethindrone (Austin, 1991). Serial cross sections reportedly showed no regional differences, and the sections adjacent to the end of the adrenal gland selected for close examination (likely the same area as the mid-GAM sections examined in our study) showed stromal expansion with no glandular or muscular differentiation (Austin, 1991). In our studies involving *in ovo* administration of estrogens and other endocrine-active compounds to alligators, we have not previously observed precocious differentiation of the oviduct. Even when using substantially higher doses of *in ovo* administered E_2, the only observed oviductal effect was increased luminal epithelial cell height (Crain et al., 1999). To our knowledge, only treatment with the ERα selective agonist PPT has induced the phenotype of precocious glandular differentiation in the alligator MD or immature oviduct.

There are several possible modes of action for why PPT and not any other ER ligands have had this effect on the alligator MD. Considering that PPT is a pharmaceutical agent, it is possible that its effect is related to its metabolism. All of the

treatments in this study were administered in a single dose at stage 19. The half-life of PPT administered to an embryonic alligator via the eggshell surface is unknown, so it may remain in circulation much longer than E_2 and other treatments, causing prolonged stimulation. The elimination half-time in serum for PPT administered subcutaneously in adult ovariectomized rats is six hours (Sepehr et al., 2012). We analyzed PPT-induced effects at stage 27, approximately 38 days after treatment. Thus, the effects of PPT in this study are not likely to be due to prolonged stimulation. Further investigation into the pharmacokinetics of PPT *in ovo* is needed.

While PPT was developed as a selective ERα agonist and was tested specifically on the alligator ERα via transactivation assay (Kohno et al., 2015), it is possible that it may have off-target effects on other steroid hormone receptors. As previously noted, *in ovo* treatment with a synthetic progestin induced oviductal hypertrophy (Austin, 1991), so it is possible that PPT may have affinity for the PGR. Expression of *PGR* was significantly increased by both 0.5 μg/g EW and 5 μg/g EW PPT treatment in our study. However, in the chicken embryo, oviductal expression of *PGR* increases upon estrogen stimulation and administration of progesterone inhibits estrogen-induced glandular differentiation in the embryonic chicken oviduct (Dougherty and Sanders, 2005; Oka and Schimke, 1969a, b; Palmiter and Wrenn, 1971). This strongly suggests an antagonistic role for progesterone in regulating estrogen stimulation in the developing avian oviduct. The developing alligator oviduct may be similarly regulated; further investigations are required to test this hypothesis.

We also investigated the expression of growth factors known to be involved in estrogen-induced proliferation in the FRT, EGF and IGF-1 (DiAugustine et al., 1988; Murphy et al., 1987), and their respective receptors. While there were no differences in *EGF* expression among the treatment groups, both 0.5 μg/g EW and 5 μg/g EW PPT treatment induced a significant increase in *IGF1* expression. The PPT-induced increase in *IGF1* and not *EGF* may be due to the more central role of IGF-1 in the proliferation of

the avian and reptilian oviduct. In the avian oviduct, oviductal *IGF1* expression increases in direct correlation with increasing oviductal size in immature Japanese quail (Fu et al., 2001) and it increases robustly following DES injection (Kida et al., 1994). Similarly, in reptiles, administration of IGF-1 via slow-release pellet induced a greater degree of oviductal proliferation in ovariectomized geckos than EGF (Cox, 1994). IGF-1 is known to be increased in the vitellogenic adult alligator oviduct (Cox and Guillette, 1993), so it is probable that IGF-1 is the primary mitogen involved in estrogen-induced oviductal proliferation in the alligator.

Taken all together, these results strongly suggest that the observed effect of PPT on the developing alligator oviduct is in fact due to its selective stimulation of ERα. Studies of the role of estrogens in the development of the FRT in mammals and birds show that the proliferative and differentiative effects of estrogen are mediated through ERα. *In ovo* exposure to DES results in rapid stromal hypertrophy and glandular formation in the chicken oviduct (Kohler et al., 1969), and the effects of DES have been shown to be mediated through ERα in the mammalian FRT (Couse and Korach, 2004; Nakamura et al., 2008). Moreover, *in ovo* exposure to PPT results in disrupted oviductal differentiation in female chickens, including hypertrophy and retention of the right oviduct, and partial oviductal development in males (Mattsson, 2008; Mattsson et al., 2011). Thus, estrogens play an important role in the normal differentiation of the avian FRT, but selective stimulation of ERα results in disrupted development. Murine studies of estrogen-regulated pathways suggest an antagonistic relationship between the estrogen receptor subtypes, with ERβ antagonizing ERα activity (Matthews and Gustafsson, 2003). PPT treatment seems to result in excess stimulation of oviductal proliferation, without ERβ signaling pathways to keep it in check.

In conclusion, stimulation of ERα *in ovo* at FPT induced significant enlargement of the alligator MD with precocious development of secretory glands and connective tissue differentiation similar to characteristics of mature adult oviduct. Increased

expression of *ER*β, *progesterone receptor*, *androgen receptor* and *insulin-like growth factor 1* might explain the phenomena in the MD. These results suggest that the embryonic alligator MD is labile to exogenous estrogen signaling through ERα, and ligands that selectively agonize ERα can disrupt normal FRT development.

Table 1. Primers for quantitative real-time PCR				
Primer name	Sequence 5' to 3'	Product Size (bp)	Annealing Temp. (°C)	Accession Number
ACTβ	GAGGGTTTTAGGTGTAACTGCTTG	195	65	NM_001286847.1, GCA_000281125.1*
	ACATACTGGCACCGCTTTTC			
AR	GCCAGACTCCTTCTCCAACC	177	62	LC012894.1
	TCTCCATCCCATGGCGAAAA			
EEF1	CGTTCTGGTAAGAAGCTGGA	169	62	XM_006269752.2
	TGACACCAACAGCAACAGTC			
EGF	GACCTGGTTTGGCCTAGTGG	243	64	XM_014593525.1
	ACGTACCCTGTTTTGGCTGG			
EGFR	ACGAAGACAAATATGCACTGG	163	62.4	XM_014602495.1
	GAGACCATCCTGTGGAATGA			
ESR1	AAGCTGCCCCTTCAACTTTTTA	71	64	NM_001287274.1
	TGGACATCCTCTCCCTGCC			
ESR2	CCAAAGAGCCCATGGTGTGA	114	64	NM_001287264.1
	ACCATTTGCAATGGGACTTGT			
IGF1	TCAGTTCGTATGTGGAGACAGA	92	64	JQ693409.1
	CCACTATCCCCTTGTGGTGT			
IGF1R	TTGTTCGATGGCAACAACAGC	230	66.4	XM_014605993.1
	GACTTTCCGGTACTCTGCCT			
PCNA	AGCAGAAGACAATGCAGACAC	198	64	XM_006267175.2
	TGCATCTCCAATATGGCTTAG			
PGR	AGCAGTTGGATTGCGCCAGAA	143	64	AB115911.1
	TCAGTGCCCGAGACTGAAGA			
RPL8	CTCTCACAATCCTGAAACCAA	115	62	XM_006266675.2
	GTTTGTCAATACGACCTCCAC			

***** primer set designed using alignment of *A. sinensis* sequence with *A. mississippiensis* genome (St John et al., 2012)

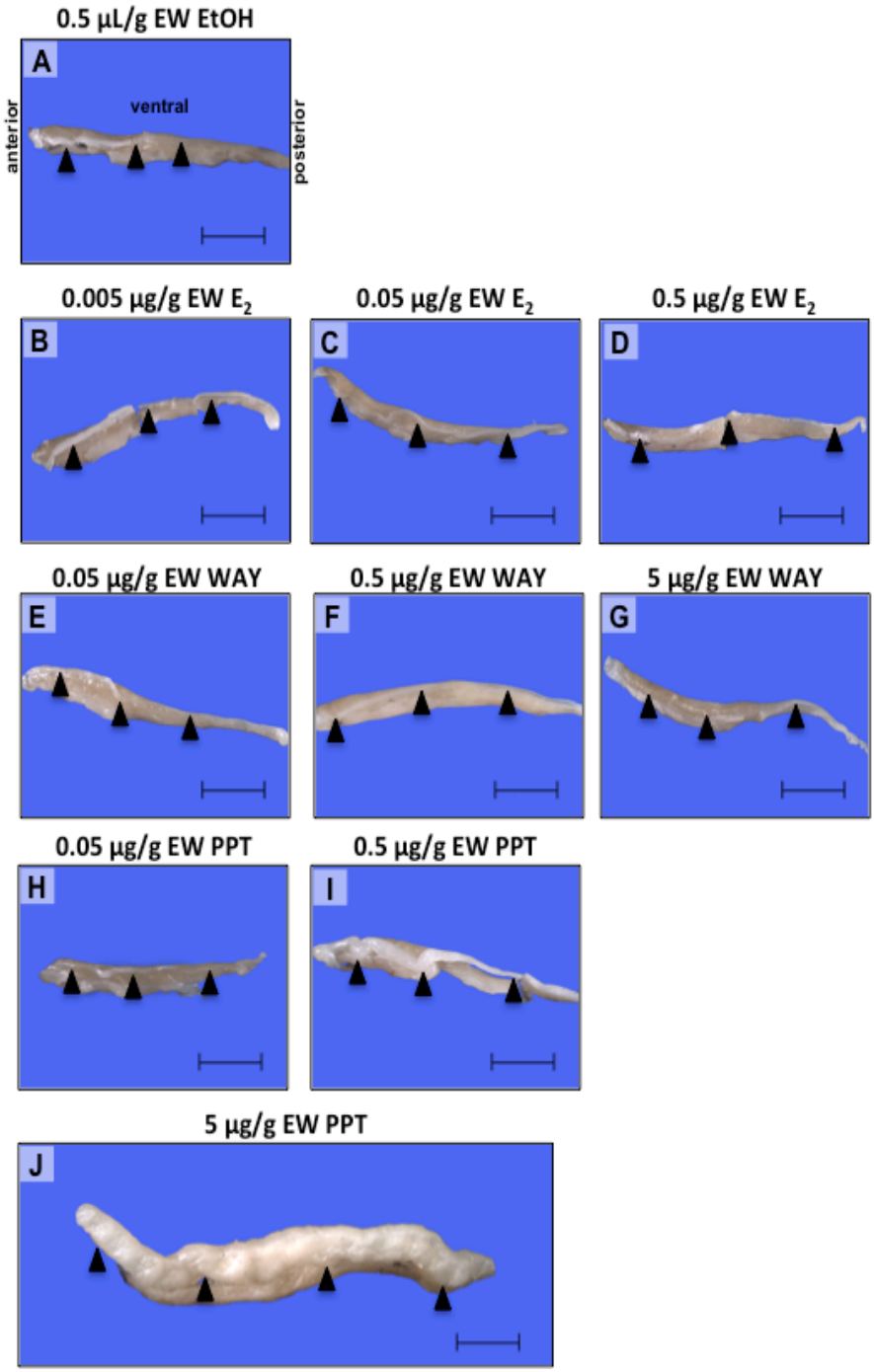


Fig. 2.1. *Gross morphology of embryonic alligator GAM complex and Müllerian duct at stage 27.* Stage 27 alligator GAM and MD treated with (A) 0.5 µL/g EW EtOH (vehicle control); (B) 0.005 µg/g EW E_2; (C) 0.05 µg/g EW E_2; (D) 0.5 µg/g EW E_2; (E) 0.05 µg/g EW WAY; (F) 0.5 µg/g EW WAY; (G) 5 µg/g EW WAY; (H) 0.05 µg/g EW PPT; (I) 0.5 µg/g EW PPT; and (J) 5 µg/g EW PPT. All scale bars indicate 3 mm. All tissues shown in lateral view; tissue orientation in (A) indicated by the terms anterior, posterior and ventral is consistent in all other images. Arrow heads indicate Müllerian duct.

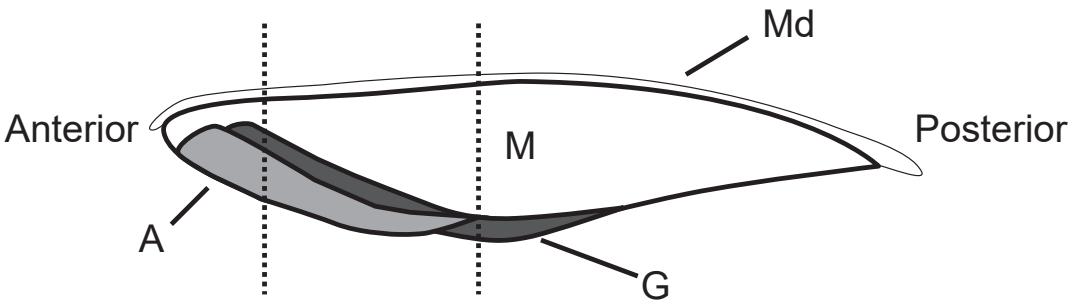


Fig. 2.2. *Schematic of embryonic alligator GAM and Müllerian duct at stage 27.*
Dorsal view of the average stage 27 alligator GAM and Müllerian duct indicating where cross sections were observed. (Modified from Deeming et al., 1988). Dashed lines indicate where along the anterior-posterior axis cross sections were examined for histological analysis. A, adrenal gland; G, gonad; M, mesonephros; Md, Mülllerian duct.

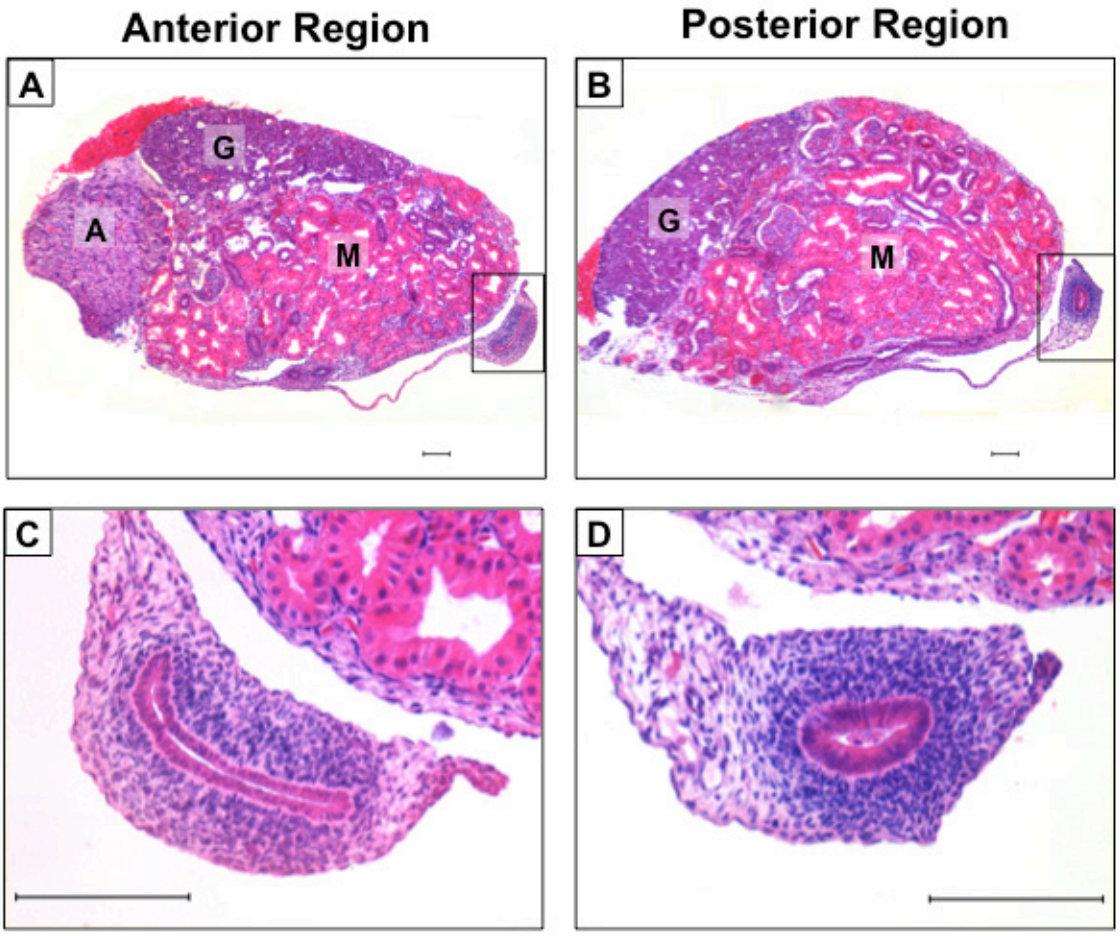


Fig. 2.3. *Histology of Müllerian duct and GAM complex at stage 27 in controls.* Representative cross sections of Müllerian duct and GAM complex at stage 27 on the anterior plane (A) and the posterior plane (B) as shown in Fig. 2. Boxed MDs in A and B are shown with higher magnification in C and D. The egg at FPT was treated with 0.5 µL/g EW EtOH (vehicle control) at stage 19. A, adrenal gland; G, gonad; M, mesonephros. Scale bars indicate 100 µm.

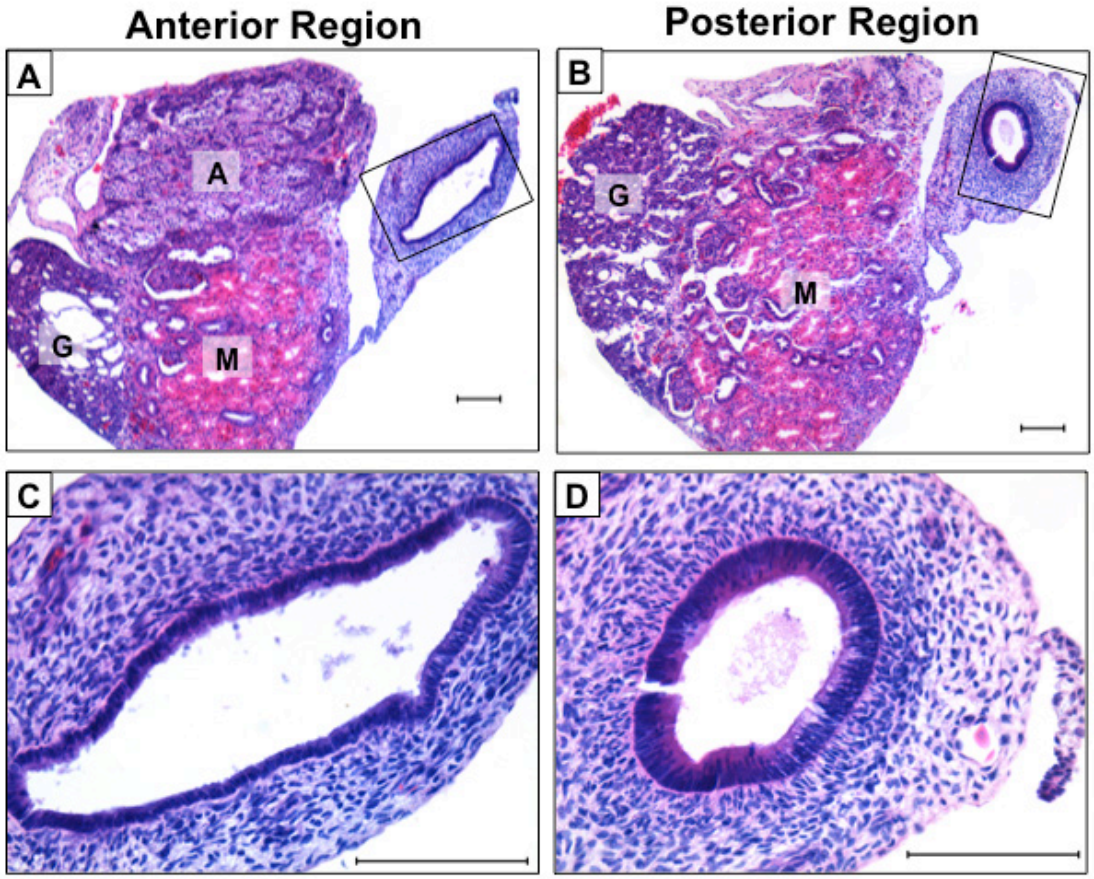


Fig. 2.4. *Histology of Müllerian duct and GAM complex at stage 27 in medium-dose PPT-treated group.* Representative cross sections of Müllerian duct and GAM complex at stage 27 on the anterior plane (A) and the posterior plane (B) as shown in Fig. 2.2. Boxed MDs in A and B are shown with higher magnification in C and D. The egg at FPT was treated with 0.5 μg/g EW PPT in EtOH at stage 19. A, adrenal gland; G, gonad; M, mesonephros. Scale bars indicate 100 μm.

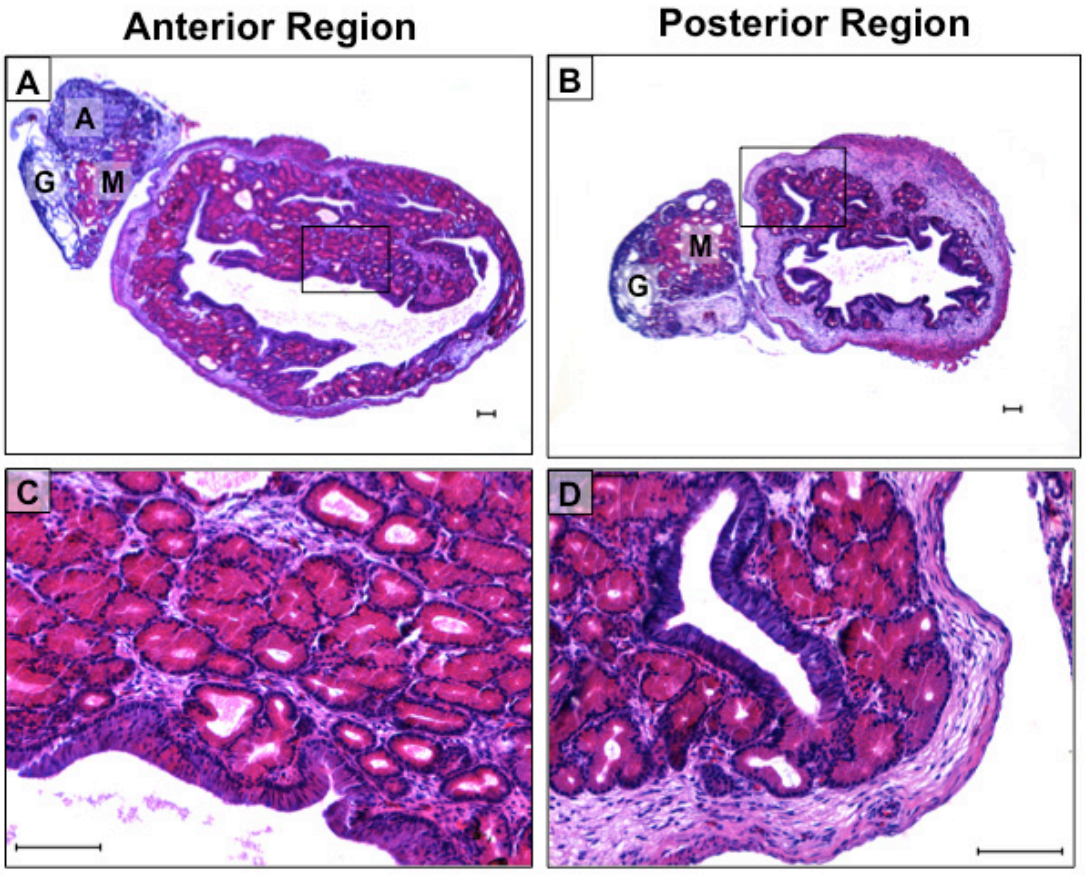


Fig. 2.5. *Histology of Müllerian duct and GAM complex at stage 27 in high dose PPT-treated group.* Representative cross sections of MD and GAM complex at stage 27 on the anterior plane (A) and the posterior plane (B) as shown in Fig. 2.2. Boxed portions of MDs in A and B are shown with higher magnification in C and D. The egg at FPT was treated with 5.0 μg/g EW PPT in EtOH at stage 19. A, adrenal gland; G, gonad; M, mesonephros. Scale bars indicate 100 μm.

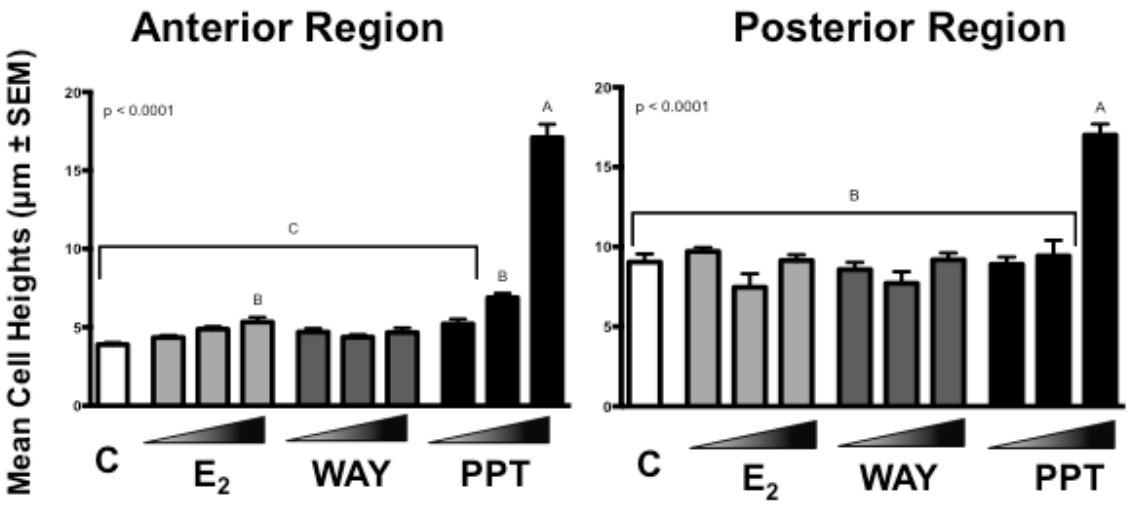


Fig. 2.6. *Luminal epithelial cell heights in the stage 27 embryonic alligator oviduct.* Mean cell heights of the MD simple columnar luminal epithelium across the treatment groups for the anterior cross sectional plane (left) and the more posterior cross sectional plane (right). Triangles under bars indicate treatment dose, low to high. Error bars indicate standard error of mean. *p*-values given for one-way ANOVA. Letters above columns indicate significantly different groups by Tukey's post-hoc test.

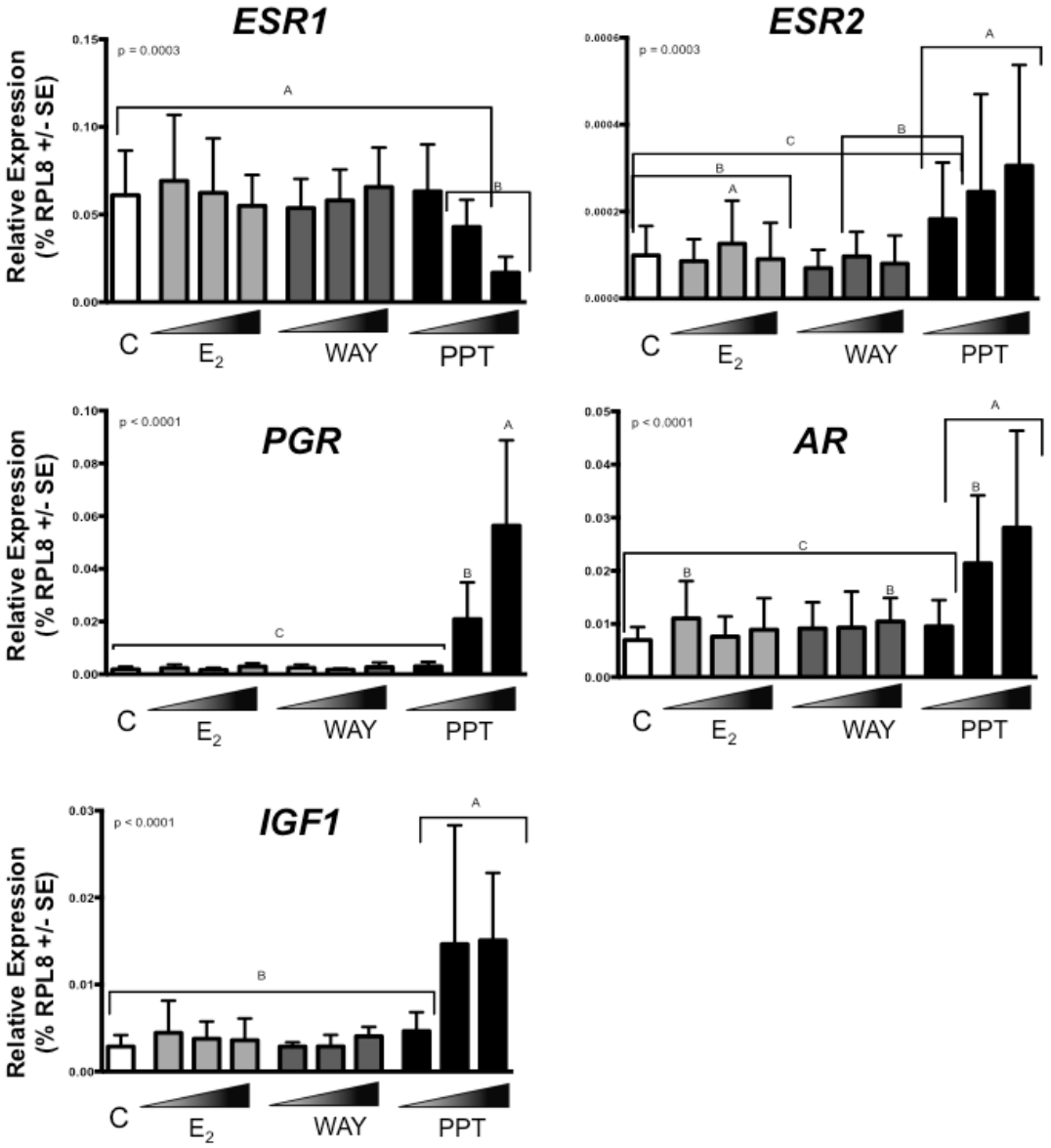


Fig. 2.7. *Expression of steroid hormone receptors and IGF-1 in alligator Müllerian duct at embryonic stage 27.* Expression values reported as relative expression normalized to ribosomal protein L8 (*RPL8*). Triangles under bars indicate treatment dose, low to high. Error bars indicate standard error mean. *p*-values based on one-way ANOVA. Letters above bars indicate significantly different groups based on Tukey's posthoc test.

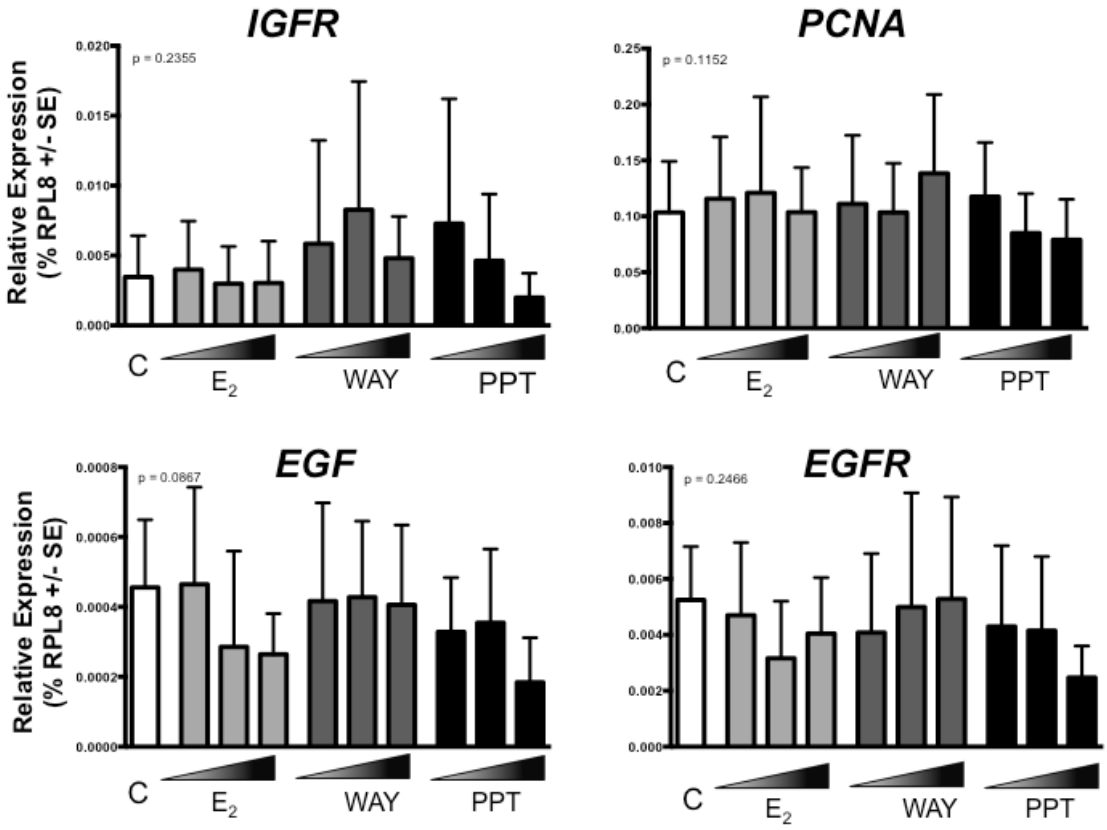


Fig. 2.8. *Expression of growth factors and proliferation marker in alligator Müllerian duct at stage 27.* Expression values reported as relative expression normalized to *RPL8*. Triangles under bars indicate treatment dose, low to high. Error bars indicate standard error mean. *p*-values based on one-way ANOVA.

CHAPTER 3:

Investigations into the contrasting effects of treatment with estradiol-17β and estrogen receptor selective agonists on the development of the alligator Müllerian duct: ligand persistence and unbalanced receptor isoform signaling

3.1. Introduction

We have previously reported that *in ovo* treatment with the ERα-selective agonist PPT induced a phenotype of precocious development in the embryonic alligator MD (ch. 2 and Doheny et al., 2016). Estrogen-induced cytodifferentiation and glandulogenesis is well-studied in the developing avian FRT (Dougherty and Sanders, 2005; Kohler et al., 1969). Based on their shared Archosaurian lineage and similar adult FRT characteristics (Palmer and Guillette, 1992), it is a reasonable assumption that the process of differentiation of MD to FRT in alligators and birds should be similar. However, estrogen-induced glandulogenesis had not previously been documented in the alligator MD. Our studies are the first to show that this phenotype can only be induced with an ERα selective agonist.

The processes of cytodifferentiation in the developing FRT of the alligator have not been previously investigated, neither following treatment with exogenous hormonally active compounds nor under normal conditions. The timepoint at which the alligator MD differentiates from a uniform structure to the regionalized structures of the mature FRT under normal conditions is not yet reported; it must occur at some point later than 1.5 years of age, after it becomes logistically difficult to maintain alligators in the laboratory. In contrast, the FRT is regionally differentiated at birth or not long thereafter in mammals (Yin and Ma, 2005) and birds (Dougherty and Sanders, 2005). Embryonic PPT treatment offers a model for investigating these processes in the alligator MD. We have shown that when PPT treatment is administered prior to the TSP, the alligator MD has developed glandular structures in a regionalized pattern by stage 27, just prior to hatchout.

Investigation of MD characteristics at several stages post-treatment could offer insight into the processes involved in this cytodifferentiation.

We have hypothesized that the effects of PPT on alligator MD are likely due to its selective activation of ERα over ERβ. Studies of estrogen receptor isoform-specific knockout mouse models suggest that ERα is necessary for proliferation and differentiation of the uterus, while ERβ plays a regulatory role, attenuating the effects of ERα (Weihua, Saji et al. 2000; Matthews and Gustafsson 2003). In our research, the differentiated alligator MD phenotype has only occurred with PPT treatment and not with E_2 treatment or treatment with ERβ selective agonist WAY, suggesting that the receptor isoforms may have similar roles in alligator FRT as in mammals. PPT treatment induced proliferation and differentiation in alligator MD, while activation of both receptors via treatment with the exogenous ligand, E_2, did not. Half maximal effective concentration (EC_{50}) values measured in receptor transactivation assays showed that E_2 is more selective for ERα than ERβ, but PPT exhibits negligible activation of ERβ (Kohno et al., 2015). Therefore, we suggest that the difference in MD phenotype following treatment with E_2 versus PPT is due to an imbalance in signaling through the ER isoforms, with PPT activating ERα in the absence of ERβ.

However, there is a second hypothesis for the differential effects of PPT and E_2 on alligator MD: that the pharmaceutical agonist PPT has a longer half-life than the native ligand. Our experiments utilized single dose administration, so a difference in metabolism of the treatment compounds could result in a difference in the duration of receptor activation. The half-life of estradiol 17-β is less than 24 hours (12-17 hours in humans) (Norris, 1997). As previously addressed in ch. 2, the half-life of PPT in adult ovariectomized rats is six hours (Sepehr et al., 2012), but the actual pharmacokinetics of this compound administered to alligator embryos via eggshell surface painting is unknown and beyond the scope of this investigation. Xenobiotic metabolism is not well-studied in wildlife in general, but it is known to often vary greatly from what

is seen in model organisms, as it is influenced by multiple factors such as body size, thermoregulation and dietary makeup (Hutchinson et al., 2014).

The goal of the following experiments was to investigate more deeply the mechanism of PPT-induced MD differentiation. We first compared the effects of PPT treatment and E_2 treatment on the alligator MD to normal male and female temperature development at four embryonic developmental stages, to better characterize the process of PPT-induced cytodifferentiation.

We tested the half-life hypothesis by comparing the effects of *in ovo* E_2 and PPT treatment on alligator MD with those of estradiol benzoate (EB), an estrogen ester. While pharmacokinetics of EB in alligator embryos are unknown, clinical studies in humans have shown that estrogen esters have a longer half-life than E_2 but similar receptor kinetics (Oriowo et al., 1980).

If an imbalance in receptor isoform signaling is a contributing factor in the PPT-induced MD phenotype, we hypothesized that treating with selective agonists for both ERα and ERβ should have a "rescue" effect on alligator MD, producing a phenotype similar to vehicle-treated controls or to that produced by E_2 stimulation. We thus compared the effects of PPT, E_2, WAY, and two combinatorial treatments of PPT and WAY on alligator MD.

3.2. Materials and methods

3.2.1. Egg collection and incubation

All experiments in this study involving alligators were carried out under the protocol approved by the Institutional Animal Care and Use Committee at the Medical University of South Carolina. Alligator eggs were collected from Lake Woodruff National Wildlife Refuge under Florida Fish and Wildlife Commission permits on 6/22/2011 and on 6/20/2013. Upon transportation to our facilities at the South Carolina Department of Natural Resources Marine Resources Research Institute, eggs were weighed, candled to

ascertain viability and placed in nest boxes of moist sphagnum moss and incubated at 30°C (FPT) in enclosed incubators. An embryo from each clutch was sampled and staged based on criteria from Ferguson (1985).

3.2.2. Treatment and tissue collection

The designs for the experiments detailed in this chapter are graphically depicted in Fig. 3.1. For the first experiment, conducted on eggs collected in 2011, once the embryos had developed to stage 19, they were randomly assigned to treatment groups: 0.5 µL/g EW ethanol, 0.5 µg/g EW E_2 (Sigma-Aldrich, St. Louis, MO, USA), or 5 µg/g EW PPT (Tocris Bioscience, Bristol, UK), doses based on previous experiments. Stock treatment solutions were prepared by dissolving compounds in absolute ethanol (95% ethanol incubated for 24 hours with molecular sieves). Eggs were weighed and treated by painting the surface with the appropriate dose of treatment solution. Embryos assigned to the FPT control group were returned to 30°C incubation, but all other treatment groups were transferred to incubation at the MPT, 33°C.

Embryos were randomly assigned to be collected at developmental stages 21, 23, 25 and 27. When the appropriate time point arrived for collection, based on a developmental equation (Kohno and Guillette, 2013), eggs were re-weighed, and embryos were weighed and sacrificed. GAM complexes, with attached MDs, were collected, one placed in RNA*later*® (Life Technologies, Grand Island, NY, USA) and one in Bouin's fixative, alternating between samples to randomize which side was used for which analysis type. The fixed tissues were incubated for 24 hours on a shaker at 4°C. The RNA*later*® -preserved tissues were then stored at -20°C. The Bouin's-fixed tissues were transferred to lithium-saturated 70% ethanol and stored at 4°C.

For the second experiment, conducted on eggs collected in 2013, embryos were incubated in the laboratory until stage 19, and then assigned randomly into treatment groups: 0.5 µL/g EW EtOH, 0.5 µg/g EW E_2, 0.5 µg/g EW EB (Sigma-Aldrich, St.

Louis, MO, USA), 5 µg/g EW WAY, 0.5 µg/g EW PPT, 5 µg/g EW PPT, or combinatorial treatments of PPT and WAY, 0.5 µg/g EW PPT + 5 µg/g EW WAY and 5 µg/g EW PPT + 5 µg/g EW WAY (see Fig. 3.1 for design schematic). Treatments were prepared and applied as described above, and all eggs were incubated at FPT for the duration of the experiment.

All embryos were sacrificed at stage 27 and GAM/MD complexes collected as described above, the right preserved in RNA*later*® and the left in 4% PFA. After overnight incubation at 4°C on a shaker, the RNA*later*®-preserved tissues were then stored at -20°C. The PFA-fixed tissues were progressively dehydrated in methanol/PBST over a 24-hour period and stored in 100% methanol at -20°C.

3.2.3. Histology

Preserved tissues were examined under a dissecting microscope and photographed using a PixeLINK digital camera and the PixeLINK Capture OEM software.

GAM/MD complexes were dehydrated through graded isopropanol and paraffin infiltrated using a Leica ASP300 autoprocessor, and paraffin embedded using a Leica EG1150C embedder. 2011 tissues were serially cross-sectioned at 7 µm using a Leica RM2255 microtome. Slides were stained using a modified Masson trichrome stain (Humason, 1979). Serial cross sections from 5 samples per treatment per stage were examined for morphological characteristics. A subset of slides from stage 27 were stained using a combination McMannus' Periodic Acid Schiff's (PAS)/ Alcian blue (AB) technique with pH 2.5 AB (Bancroft and Gamble, 2008) to examine histochemical profiles.

The 2013 samples were serially cross-sectioned or serially lateral-sectioned at 8 µm using a Leica manual rotary microtome, and stained with hemotoxylin and eosin using a Leica Autostainer XL with a Leica CV5030 coverslipper.

For each treatment group, five samples were examined in full, 2-3 as serial cross sections and 2-3 as serial lateral sections.

Representative digital photomicrographs were taken with a ProgRes Speed XT core 5 camera (Jenoptik, Jena, Germany).

3.2.4. Sexing of embryos

At stages 25 and 27, the embryos were sexed based on gonadal characteristics, as described by Smith and Joss (1993). Stage 21 and 23 gonads were too ambiguous for definitive sexing. Two stage 27 MPT- incubated vehicle control embryos were classified as female based on gonadal characteristics, so they were removed from all further analysis to not skew results.

3.2.5. RNA isolation, reverse transcription and quantitative PCR

For the 2011 samples, RNA isolation and reverse transcription and QPCR were performed according to methods detailed in chapter 2. RNA*later*® -preserved MD tissues were manually separated from GAM complexes under a dissecting microscope and stored at -20°C until RNA extraction. For three of the stage 27 vehicle control MPT samples, MD was fully or partially regressed, so these samples were omitted from further analysis. Whole MDs were homogenized and RNA isolated from tissues using the acid phenol-guanidinium thiocyanate-chloroform extraction method followed by further purification using the SV Total RNA Isolation System with DNase-I treatment (Promega, Fitchburg, WI, USA). RNA concentration was measured using a Nanodrop Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and quality was assessed via denaturing agarose gel electrophoresis. Prior to cDNA synthesis, RNA was diluted to matching concentrations and used as a template for quantitative real-time PCR to check for genomic DNA contamination. RNA was reverse transcribed using the iScript cDNA

Synthesis kit (Bio-Rad, Hercules, CA, USA) per manufacturer's protocol with 15 µL diluted RNA in 20 µL total reaction volume, and the resulting cDNA was diluted 1:5 in nuclease-free water.

This protocol was slightly modified for the 2013 samples. Whole MDs (n=8 per treatment group) were homogenized for RNA extraction via the SV Total RNA Isolation System with DNase-I treatment (Promega). RNA concentration and quality was assessed as described, then RNA was diluted to matching concentrations. Reverse transcription was performed using the High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems, Foster City, CA, USA) per manufacturer's protocol, using 10 µL diluted RNA in 20 µL total reaction volume, and the resulting cDNA was diluted 1:5 in nuclease-free water.

QPCR was performed using a CFX96 real-time system (Bio-Rad). Reactions were performed in triplicate using 0.01 U/µl AmpliTaq Gold (Applied Biosystems, Carlsbad, CA, USA) and an in-house SYBR green reaction mixture described in chapter 2, and 1/25 volume diluted cDNA. The thermal cycler program consisted of an initial denaturation step (95°C, 5 min) followed by 45 cycles of denaturation (95°C, 15 s) and annealing and elongation at an optimized temperature (45 s). After the final cycle, a dissociation curve was generated for each reaction to assess the reaction specificity via an incremental heating step (+0.5°C/5 s).

Primer sequences, product size and optimal annealing temperatures are listed in Table 1 (pg. 28). Primer specificity was assessed as described in chapter 2.

3.2.6. Statistical analysis

QPCR data were collected and analyzed using CFX Manager version 3.0 software. For the 2011 samples, three internal control genes, *EEF1*, *ACTB*, and *RPL8,* were assessed, but no single internal control gene was appropriate for use as a normalization factor, as all three showed significant effects of treatment via one-way

ANOVA. For normalization of QPCR values, a normalization factor (NF2) was calculated based on the geometric mean of all three internal control genes using the geNORM analytical tool (Vandesompele et al., 2002). Absolute copy number of each target gene was divided by NF2 and reported as relative expression. These normalized expression values were used to conduct one-way ANOVA to calculate the effect of treatment on target genes at each of the four developmental stages, and outlying values were removed via ROUT method with a maximum false discovery rate set at 1%. Significance was set at $p < 0.05$, and a Tukey's multiple comparison post-hoc test was used to detect differences among treatment groups.

For the 2013 sample set, expression of *ACTB* was not significantly affected by treatment via one-way ANOVA, so expression values of all other genes of interest were normalized to *ACTB*. Data were log-transformed to achieve homogeneity of variance. One-way ANOVA was utilized to calculate the effects of treatment on expression levles of genes of interest. Outlying values were removed by examination of a residual plot and ROUT method with a maximum false discovery rate set at 1%. Significance was set at $p < 0.05$, and a Tukey's multiple comparison post-hoc test was used to detect differences among treatment groups.

All statistical analysis was performed using both JMP12 and Graphpad Prism 6 software.

3.3. Results

*3.3.1. Treatment effects on morphological characteristics of M*üllerian duct

As seen previously (Kohno et al., 2015), PPT treatment at MPT induced significant MD enlargement, while E_2 treatment did not. This enlargement was already apparent at stage 23 and became increasingly more pronounced at later stages (Fig. 3.2). At stage 27 in the MPT vehicle control samples, complete MD regression was observed in two samples, and partial MD regression in 5 samples.

For the 2013 samples, the morphological characteristics of MD of the vehicle control samples and those treated with 0.5 µg/g EW E_2, 5 µg/g EW WAY, and the 0.5 µg/g EW and 5 µg/g EW doses of PPT at FPT were identical to what has previously been documented for these treatments in chapter 2. There were no observable gross morphological differences between MD treated with E_2 or EB.

Contrary to our hypothesis, the co-treatment with WAY did not alter the PPT-induced MD phenotype. The combined 0.5 µg/g EW PPT + 5 µg/g EW WAY treated MDs were similar to those treated with 0.5 µg/g EW PPT, somewhat enlarged compared to the controls. The combined 5 µg/g EW PPT + 5 µg/g EW WAY treated MDs were similar to the 5 µg/g EW PPT treated MDs, massively hypertrophied compared to the controls. The hypertrophied MDs from these treatment groups were weighed prior to RNA extraction for proper calculation of reagents, and there was no difference in mean MD mass (5 µg/g EW PPT mean = 51.38 7.84 mg, 5 µg/g EW PPT + 5 µg/g EW WAY mean = 43.25 6.05 mg, two-tailed t-test $p = 0.43$).

3.3.2. Histological characteristics of the gonads and determination of sex

For the 2011 samples, gonadal morphology was examined at stages 25 and 27 to sex embryos. At stages 21 and 23, gonadal morphology was too ambiguous to determine sex. The defining characteristics for identifying testis differentiation in alligator are well-defined medullary sex cords with enlarged cells, and the defining characteristics for ovarian differentiation are lacunae in the medulla and a well-defined cortex with germ cells (Smith and Joss, 1993). Treatment with E_2 and PPT induced ovarian formation at MPT in all stage 25 and stage 27 embryos. E_2-treated gonads were morphologically similar to those of the FPT vehicle control samples, but PPT-treated gonads showed structural differences, with larger lacunae and less cortical definition than controls and E_2-treated gonads (Fig. 3.3).

As described in the methods section, all but two of the MPT stage 25 and stage 27 vehicle control gonadal samples showed well-defined sex cords, the main testicular characteristic identifiable at these stages (Fig. 3.3). These stage 27 MPT control samples with ovaries were removed from further analysis. This and other incidences of females occurring despite incubation at MPT throughout the canonical TSP inspired a study in our research group that determined the window of thermosensitivity in alligators is longer than previously documented (McCoy et al., 2015). The feminized males in this study were from a clutch collected from the field at an earlier developmental stage and thus held longer at the FPT than others, which likely influenced their gonadal sex determination in a female direction.

3.3.3. Treatment effects on Müllerian duct histology

For the 2011 samples, MD structure was similar for FPT control samples and MPT E_2-treated samples across all the stages examined, displaying the simple columnar luminal epithelium and fibroblastic mesenchymal stroma previously characterized in chapter 2.

At stage 21, the MPT PPT-treated samples were not distinctly different histologically from the FPT controls, aside from enlarged size due to some stromal expansion (Fig. 3.4). By stage 23, this stromal expansion in the MPT PPT-treated MDs was much more apparent and involved hypertrophy of the stromal cells due to imbibition (Fig. 3.4). At stage 25, there was glandular formation along the luminal epithelium of these MPT PPT-treated MDs (Fig. 3.4).

The stage 27 MPT PPT-treated tissues displayed the same characteristics documented in ch 2. With the use of modified trichrome stain, the formation of connective and muscular tissues in the periphery of the stroma was evident, as connective tissue stains green and muscular tissue red (Fig. 3.4). At stage 27 in the MPT control

samples, complete MD regression was observed in two samples and partial MD regression in 5 samples.

As with the FPT samples characterized in ch. 2, stage 27 MPT PPT-treated MD in this experiment showed anterior-posterior regionality. The epithelium of more anterior regions of the MD was expanded with glands (Fig. 3.5A), while more posterior regions had fewer glands and more connective tissue and smooth muscle differentiation (Fig. 3.5 B). These regions also stained differently with AB/PAS. In the more anterior region, the luminal epithelium is positive for AB, while the glands are PAS positive (Figs. 3.5 C and 3.5 E), and in the more posterior region, the staining was less intense in general but the epithelium was positive for both AB and PAS, while the glands were only AB positive (Figs. 3.5 D and 3.5 F).

In the 2013 samples, as seen with morphological characteristics, the histological characteristics of previously examined treatments recapitulated what has been previously described in chapter 2. The EB-treated MD displayed similar characteristics to the E_2-treated and control tissues. There were no observable histological differences between 0.5 µg/g EW PPT-treated MD and the MDs treated with combined 0.5 µg/g EW PPT + WAY, and no observable differences between the 5 µg/g EW PPT-treated MDs and combined 5 µg/g EW PPT + WAY samples.

Lateral sectioning of a subset of samples confirmed previous observations on anterior-posterior regional differences in the stage 27 alligator MD treated with 5 µg/g EW PPT at FPT (Fig. 3.6). At the anterior end, the stroma of the enlarged MD was densely packed with secretory glands. Moving posteriorly, there were fewer glands and the stroma was made up of irregular cells and loose connective tissue.

3.3.4. Treatment effects on expression of key genes in Müllerian duct

In the 2011 sample set, at MPT, PPT affected expression of key genes in MD at all stages sampled. For the steroid hormone receptors (SHRs) analyzed, overall, PPT

treatment resulted in decreased expression of *ESR1* and increased expression of *ESR2*, *PGR*, and *AR* (Fig. 3.7). At stage 21, *ESR1* expression was significantly reduced in PPT-treated MD compared to FPT controls, but not compared to E_2-treated MD. At stages 23 and 25, however, *ESR1* expression was similar in MPT and FPT controls and E_2-treated MD, while it was significantly decreased in PPT-treated MD. At stage 27, this trend was not quite as extreme, but *ESR1* expression was still significantly lower in PPT-treated tissues than in FPT controls.

ESR2 expression was very low in MD across all stages and treatments, and at stage 21 there were no significant differences in expression among the treatments. In MPT PPT-treated MD, *ESR2* expression increased over the developmental time course, and was significantly higher than FPT controls at stages 23, 25 and 27. *PGR* expression was significantly higher in PPT-treated MD than all other treatments at every stage sampled. Expression of *AR* was significantly higher in PPT-treated tissues than all other treatments at stages 21, 23 and 25, but at stage 27 *AR* expression in PPT-treated MD decreased to a level similar to E_2-treated and MPT vehicle control MDs.

Other key genes of interest analyzed were the growth factors *EGF* and *IGF1*, and proliferation marker *PCNA* (Fig. 3.8). There were no statistically significant differences in *EGF* expression across the treatments at stages 21, 23, or 27, but at stage 25 it was significantly reduced in PPT-treated tissue. *IGF1* was significantly increased in PPT-treated MD compared to all other treatments at stages 21, 23 and 25, and at stage 27 it was significantly higher than in FPT control MD but not compared to MPT control or E_2-treated tissues. Differences in *PCNA* expression were only observed at stage 25, when it was significantly higher in PPT-treated tissue compared to MPT controls but not to FPT controls or E_2-treated MD.

The gene expression results for the 2013 samples at FPT follow similar patterns to what has previously been seen with these treatments (Fig. 3.9). When directly comparing the effects of EB vs. E_2 on the expression of the transcripts assessed, the only significant

difference was with *PGR,* for which the expression level is nearly 2.5-fold greater with EB treatment than E_2 treatment. EB treatment also increased transcript levels of *ESR2* and *AR* slightly more than E_2, although this was not statistically significant (Fig. 3.9).

The combination treatments, adding WAY to PPT, were expected to attenuate the effects of PPT treatment alone, but this did not appear to be the case at the level of SHR gene expression. Effects of adding 5 µg/g EW WAY to 0.5 µg/g EW PPT and 5 µg/g EW PPT on the expression levels of the genes investigated were not significantly different than these doses of PPT alone on *ESR1, ESR2, PGR* and *AR.* There was, however, a significant difference between the treatments in *IGF1* expression. There was nearly a twofold decrease in *IGF1* in the combination treatments compared to the PPT treatments alone.

3.4. Discussion

The multi-stage sampling experiment provided a more in-depth look at the process of MD differentiation induced via PPT treatment. Alligator embryos develop from stage 19, the time of experimental dosing, to stage 21, the first sampling time point, in just 5 days at MPT (Kohno and Guillette, 2013), and already at this point PPT-treated MDs displayed significant differences in the expression of key genes. There were no observable morphological or histological changes in PPT MDs at this stage, but PPT-induced MD phenotypic differences became more exaggerated as development progressed.

The pattern of histological changes in the PPT-treated MD is similar to what has been seen in foundational studies on the differentiation of the avian oviduct, in which immature chick oviduct was treated with exogenous estrogens. Within 24 hours of subcutaneous injection with DES, the left oviductal magnum of 5-day-old chicks demonstrates substantial stromal hypertrophy due to edema (Kohler et al., 1969), similar to the stromal expansion seen at stage 23 in PPT-treated alligator MD.

In DES-treated chick luminal epithelium, the mitoses increase and the cells elongate, nuclei enlarge and nucleoli hypertrophy, and the cells differentiate into ciliated and goblet secretory cells. Secretory glands appear by day 4 as invaginations of the epithelium and by day 9 these glands expand into the stroma, while stromal cells at the periphery of the MD differentiate into bundles of smooth muscle (Kohler et al., 1969). The precocious glandular formation in PPT-treated alligator was first detectable at stage 25, in which a small number of glands appear, forming from invagination of the luminal epithelium. At stage 27, these glands expand into the stroma in anterior regions of the MD, and the periphery of the MD shows smooth muscle differentiation.

Differential histological staining utilized in this experiment supports the observations of regionalized differentiation of the PPT-treated MD. At stage 27, the PPT-treated alligator MD stained positively with AB and PAS, indicating the presence of mucopolysaccharide secretory products. PAS stains tissues magenta in the presence of a variety of mucin polysaccharides, particularly glycosaminoglycans, while AB selectively stains acidic mucins (Bancroft and Gamble, 2008). In the adult alligator oviduct, secretory products vary regionally and with reproductive status. The secretory epithelia stains positively with AB for glycosaminoglycans in a regional fashion: staining is most intense in the tube, slightly less intense in the anterior uterus and very limited in the posterior uterus (Palmer and Guillette, 1992). A similar pattern was seen in the stage 27 PPT-treated MD: the luminal epithelium was positive for AB, and this was seen most clearly in the anterior region.

In reproductively active female alligators, between mating and oviposition, the oviductal luminal epithelium of the tube and anterior uterus, as well as the tubal glands, stain positively for PAS (Bagwill et al., 2009). PAS staining was not detected very strongly at all in the stage 27 PPT-treated MD epithelium, but it was strong in the glands of the anterior region, decreasing posteriorly. Taken together, the AB/PAS staining patterns seen in stage 27 PPT-treated alligator MD share some similarities

to those in reproductively active adult alligator FRT. This supports the idea that PPT-induced cytodifferentiation in the embryonic alligator MD follows a regionalized pattern reminiscent of the highly regionalized adult FRT.

These histological similarities do not necessarily indicate that PPT-induced MD differentiation exemplifies the normal process of MD differentiation in the alligator. The hypertrophy of the PPT-treated MD stroma is indicative of edema, and smooth muscular differentiation of this tissue is highly disorganized. There are also clear irregularities in the glandulogenesis of the epithelium. Until normal FRT differentiation in alligators can feasibly be investigated, however, the PPT-treated MD model still provides valuable information about this process.

On a molecular level, SHR expression is rapidly affected by PPT treatment in a manner consistent with feedback regulation discussed in chapter 2. Already by stage 21, PPT treatment results in significantly decreased expression of *ESR1,* and increased expression of *PGR* and *AR* in alligator MD compared with controls and E_2 treatment. Interestingly, at stage 21 *ESR1* and *AR* are strongly expressed in control MD at both MPT and FPT, while *ESR2* and *PGR* and have little to no expression in control MD. Both PPT and E_2 treatments result in increased expression of *PGR* and A*R* at stage 21, though E_2 expression activation is to a much lesser degree. This trend continues at later developmental stages, supporting the hypothesis that the effects of PPT treatment on alligator MD are due to its selective ERα activation.

PCNA is a DNA polymerase-associated protein used as a marker of cellular proliferation (Helguero et al., 2005; Krishna et al., 1994). *PCNA* expression has been used as a proliferative marker in juvenile alligator oviduct, when it was shown to increase following treatment with follicle stimulating hormone (FSH) (Moore et al., 2012). There were no differences in *PCNA* expression in the experiment detailed in ch.2, despite the dramatic size increase in PPT-treated MD compared with other treatment groups. As these tissues were only sampled at stage 27, it was hypothesized that PPT-induced cellular

proliferation occurred prior to stage 27. However, in this experiment, the only *PCNA* expression differences were seen at stage 25, and they weren't as dramatic as would be expected. Based on just this marker, the role of active proliferation in the PPT-induced MD phenotype and the time point at which it occurs remains to be elucidated.

The experiment involving the 2013 sample set, designed to further elucidate the hypothesis that the PPT-induced phenotype is due primarily to selective ERα activation, does not provide as much insight as expected. The effects of EB closely matched those of E_2, and the effects of PPT treatment seen in the multi-stage experiment occurred relatively rapidly following administration, suggesting that the effects of PPT are not due to this pharmaceutical agonist having a longer half-life than endogenous estrogen. The lack of attenuation of the PPT treatment-induced effects by co-treatment with WAY contradicts the proposed hypothesis attributing the PPT-induced MD phenotype to imbalanced signaling between the ER isoforms. This result may be due to factors related to the experimental design, however, such as the timing of dose administration and the doses themselves.

At stage 19, when the treatments were administered, *ESR2* transcript levels are not detectable via QPCR, and levels of *ESR2* remain much lower than those of other SHRs throughout embryonic development, as shown in FPT controls in the staged experiment. It is therefore possible that ERβ levels in the alligator MD are too low for their selective activation with WAY to have downstream signaling effects strong enough to regulate the effects of ERα activation via PPT.

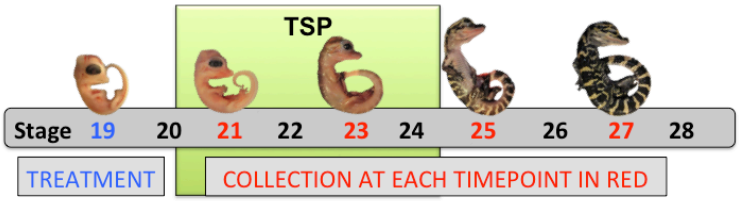
While the pharmaceutical agonists used in this experiment were selected due to their specificity for ERα and ERβ via receptor transactivation assay *in vitro* (Kohno et al., 2015), the doses utilized were based on the receptor kinetics of the agonists on each single receptor isoform in isolation, rather than the co-existence of both of the ER isoforms *in vivo*. Taken alongside the observed differences in receptor isoform expression levels in the embryonic MD, it is highly likely that the dosage of WAY used here was not

enough to induce a level of ERβ activation that could counter the highly activating effects of PPT on ERα.

Even so, the effects of combinatorial treatment on *IGF1* expression provide some support for the concept of a regulatory role of ERβ on ERα-mediated proliferation in the alligator MD. The attenuated *IGF1* levels made no difference in the PPT-induced hypertrophy, however, but these transcripts were only measured at stage 27. The multi-stage experiment showed that PPT-induced hypertrophy begins much earlier, and PPT-induced increases in *IGF1* levels are more dramatic at earlier developmental stages, while transcript levels of *ESR2* increase over developmental time. An experiment incorporating a later dosage time, or multiple doses over a developmental time course, might better elucidate the interplay between ERα and ERβ signaling in proliferative and differentiative processes in alligator MD.

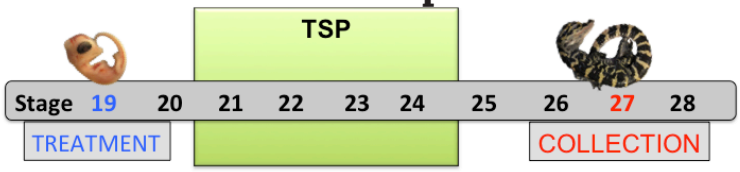
Taken together, the results of these experiments support the value of the PPT-induced MD phenotype as a tool for investigating the role of estrogen signaling in the developing alligator FRT, even if the different effects of PPT treatment and E_2 treatment may be due to some unknown aspect of PPT metabolism in the embryonic alligator. The response of embryonic alligator MD to PPT follows a similar pattern to the response of the developing avian FRT to estrogens. This response, particularly at a molecular signaling level, provides insight into the role of estrogen signaling in developing alligator FRT, but further experiments are needed to elucidate the specific roles of the estrogen receptor isoforms in proliferation and differentiation in the developing alligator FRT.

2011 Samples



Temperature	Treatment	Dose
FPT (30.0° C)	EtOH	0.5 µl/g EW
MPT (33.0°C)	EtOH	0.5 µl/g EW
	E_2	0.5 µg/g EW
	PPT	5 µg/g EW

2013 Samples



Temperature	Treatment	Dose
FPT (30.0° C)	EtOH	0.5 µL/g EW
	E_2	0.5 µg/g EW
	EB	0.5 µg/g EW
	WAY	5 µg/g EW
	PPT	0.5 µg/g EW
		5 µg/g EW
	PPT + WAY	0.5 µg/g EW + 5 µg/g EW
		5 µg/g EW + 5 µg/g EW

Fig. 3.1 *Schematic of experimental designs.*
Time points of dosing and collection relative to the thermosensitive period (TSP) indicated at top; treatments and dosage indicated in charts. Top: 2011 experiment, bottom: 2013 experiment.

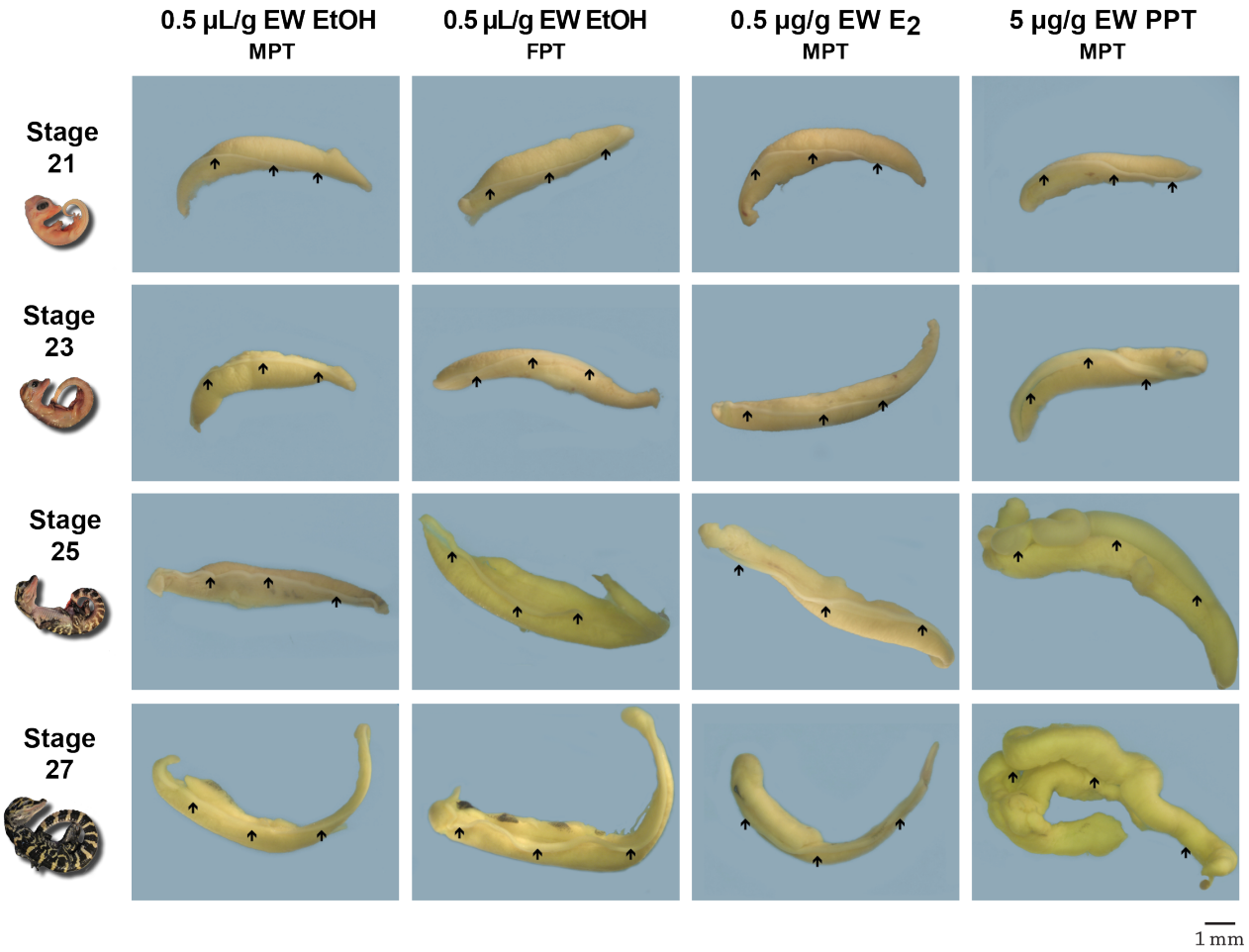


Fig. 3.2. *Effects of treatment on embryonic alligator Müllerian duct morphology at four developmental time points.* Representative photographs of embryonic alligator GAM with attached MD. Treatment and incubation temperature indicated above images, from left to right: 0.5 µL/g EW EtOH at MPT, 0.5 µL/g EW EtOH at FPT, 0.5 µg/g EW E_2 at MPT and 5 µg/g EW PPT at MPT. Stage of collection indicated at left of images, from top to bottom: 21, 23, 25 and 27. Arrows indicate MD.

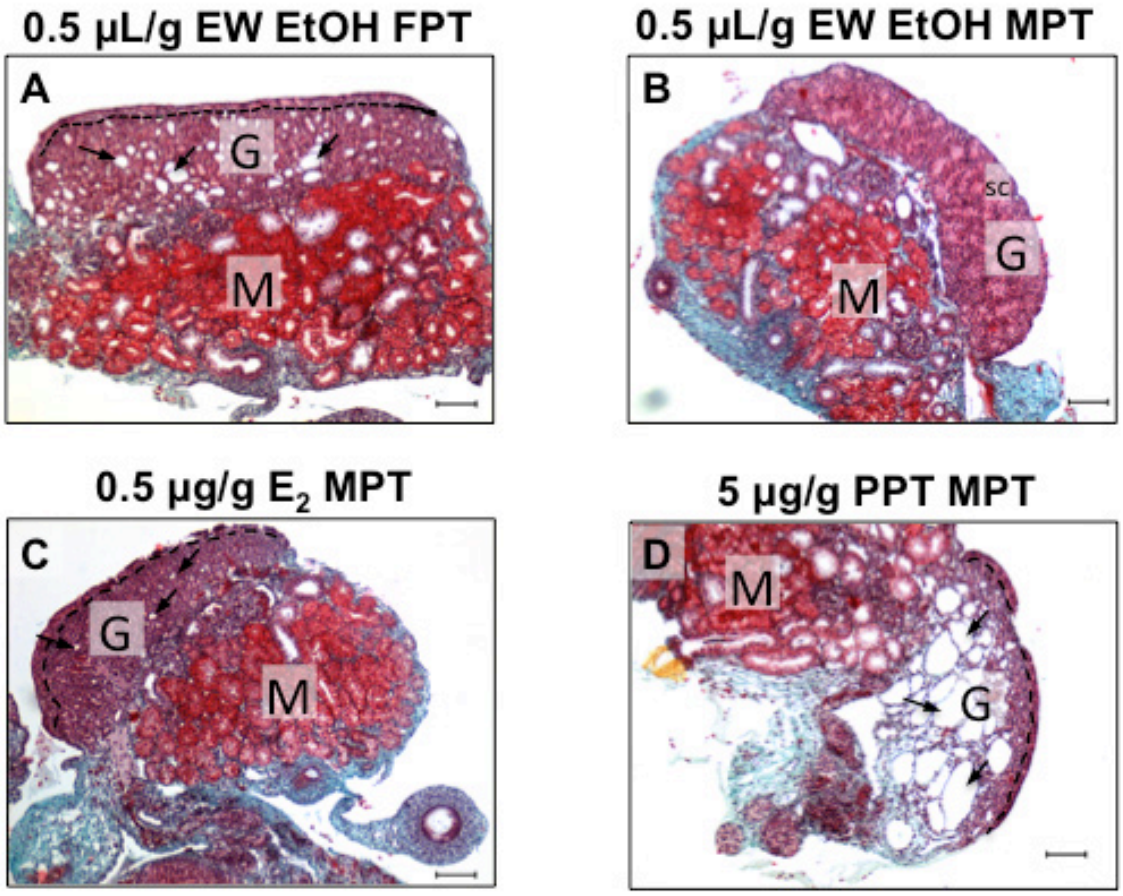


Fig. 3.3. *Effects of treatment on gonadal histology at stage 27.*
Representative cross sections of stage 27 alligator GAM complexes.(A) 0.5 µL/g EW EtOH at FPT. (B) 0.5 µL/g EW EtOH at MPT. (C) 0.5 µg/g EW E_2 at MPT. (D) 5 µg/g EW PPT at MPT. G = gonad, M = metanephros. sc = sex cords. Arrows indicate ovarian lacunae. Dashed line indicates base of ovarian cortex. Scale bars = 100 µm.

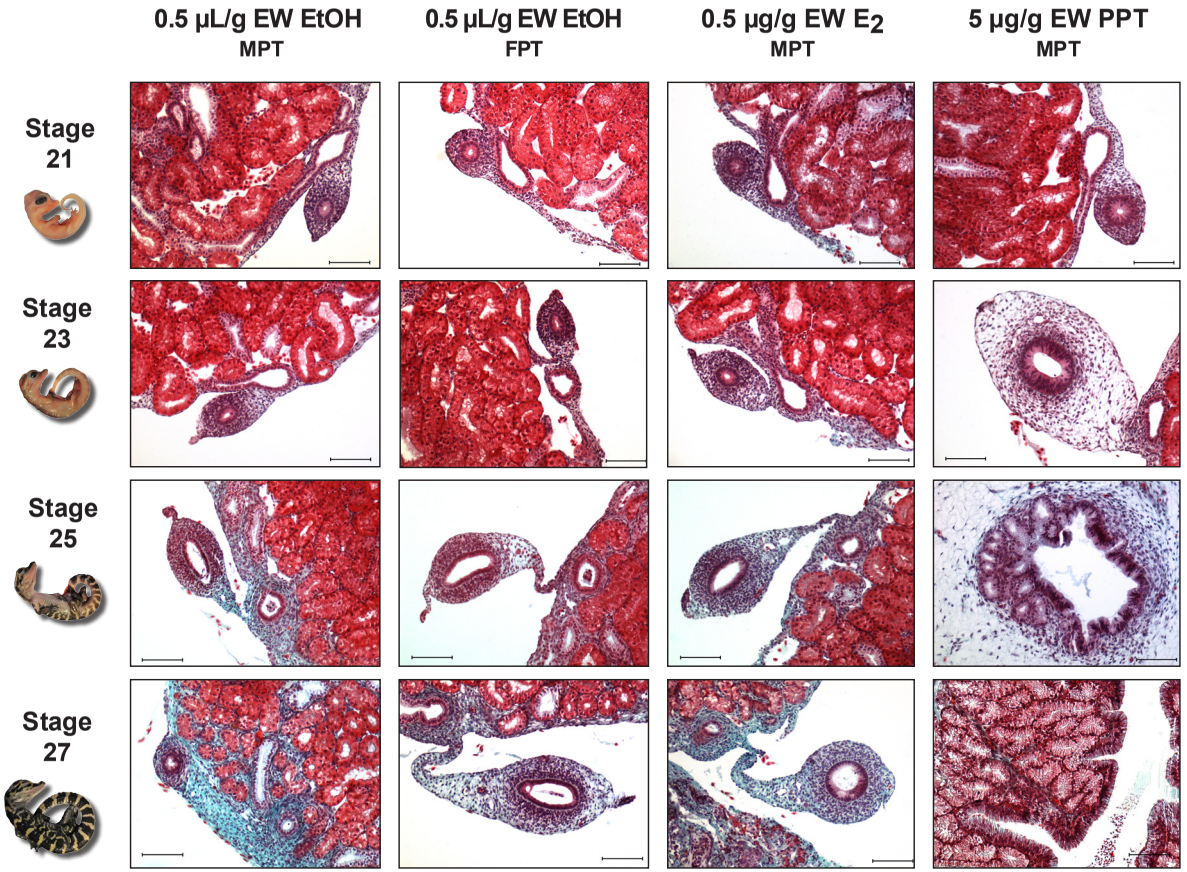


Fig. 3.4. *Effects of treatment on Müllerian duct histology over four developmental stages.* Representative cross sections of embryonic alligator MD. Treatment and incubation temperature indicated above images, from left to right: 0.5 µL/g EW EtOH at MPT, 0.5 µL/g EW EtOH at FPT, 0.5 µg/g EW E_2 at MPT and 5 µg/g EW PPT at MPT. Stage of collection indicated at left of images, from top to bottom: 21, 23, 25 and 27. Scale bars = 100 µm.

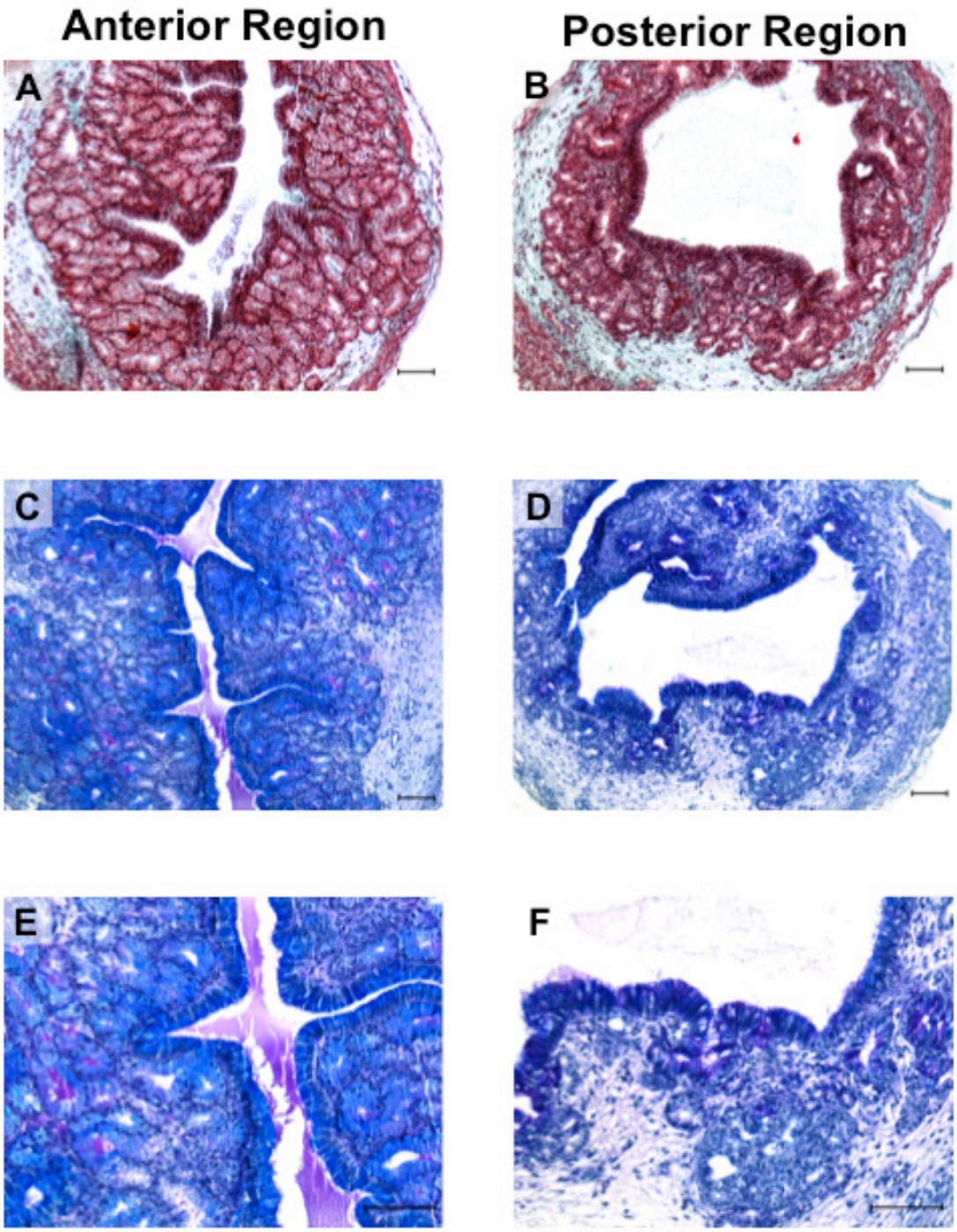


Fig. 3.5. *Regionalized histological and histochemical characteristics of stage 27 PPT-treated embryonic Müllerian duct.* Representative cross sections of stage 27 5 μg/g EW PPT-treated MPT MD. (A) Trichrome stained anterior region of MD. (B) Trichrome-stained, more posterior region of MD. (C) and (D): similar regions stained with AB/PAS. (E) and (F): magnifications of (C) and (D), respectively. Scale bars = 100 μm.

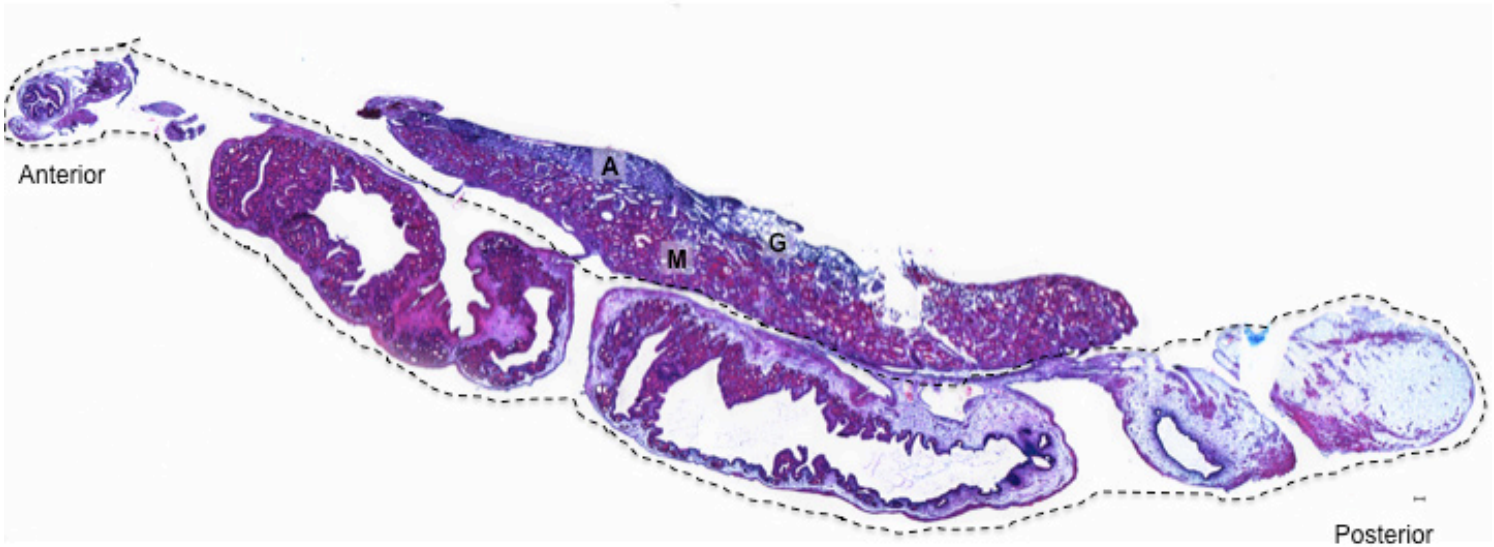


Fig. 3.6. *Lateral view of regionalized histological characteristics of stage 27 PPT-treated Müllerian duct.* Representative lateral section of stage 27 5 μg/g EW PPT-treated FPT embryonic GAM and MD, stained with H&E. A = adrenal gland, M = mesonephros, G = gonad. Dashed line encompasses MD. Anterior – posterior axis indicated. Scale bar = 100 μm.

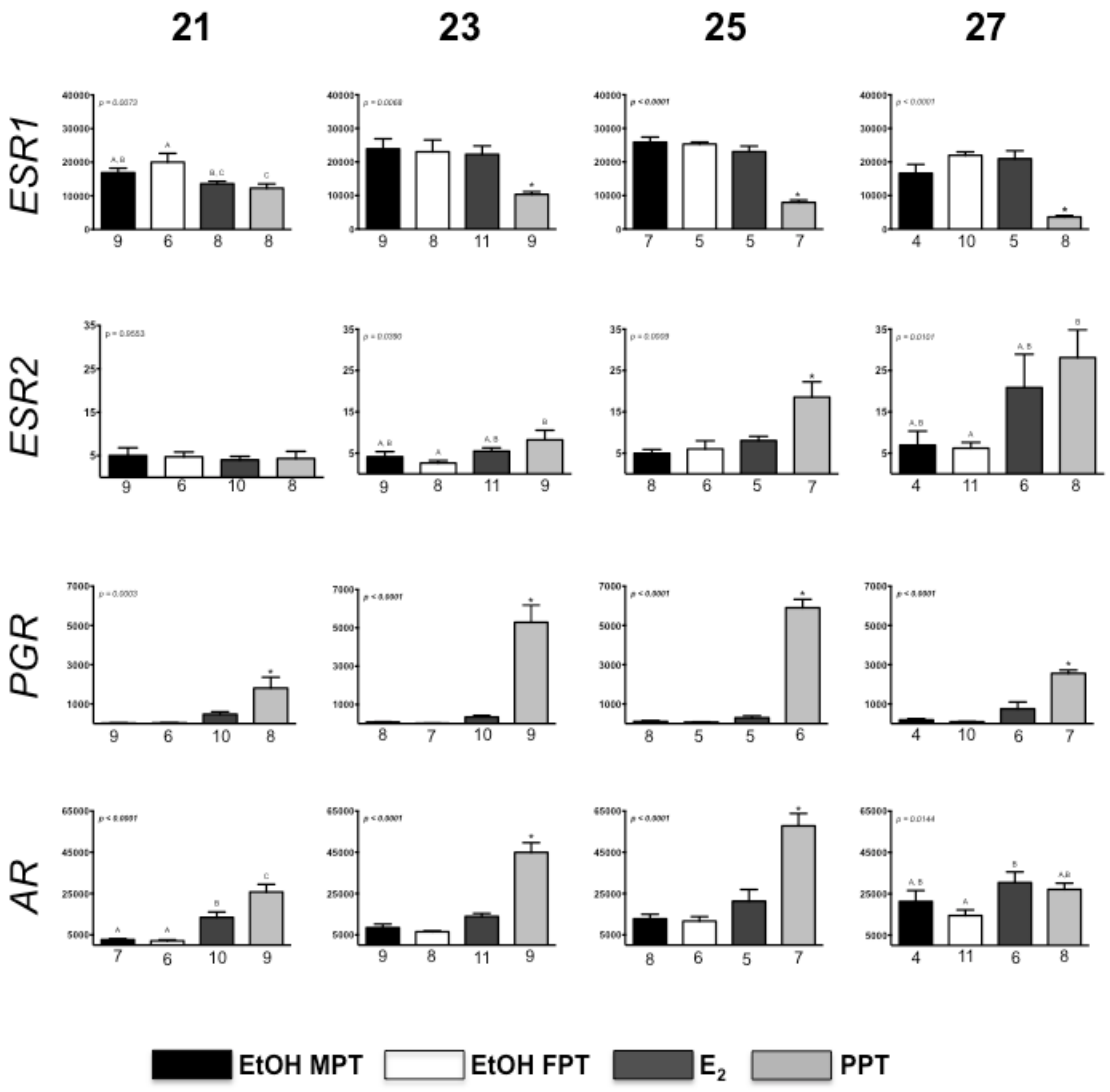


Fig. 3.7. *Effects of PPT treatment on steroid hormone receptor expression in Müllerian duct at four developmental stages.*

Relative expression of SHRs normalized to calculated normalization factor. Error bars indicate mean standard error. Genes indicated to left of graphs, from top to bottom: *ESR1*, *ESR2*, *PGR*, *AR*. Stage of collection above, from left to right: 21, 23, 25, 27. Bar color indicates treatment: black = 0.5 µl/g EW EtOH, MPT; white = 0.5 µl/g EW EtOH, FPT; dark gray = 0.5 µg/g EW E_2; light gray = 5 µg/g EW PPT. *p*-values via one-way ANOVA indicated above graphs. Sample size indicated below bars. Letters above bars indicate differences among treatment groups per Tukey's multiple comparison post hoc test.

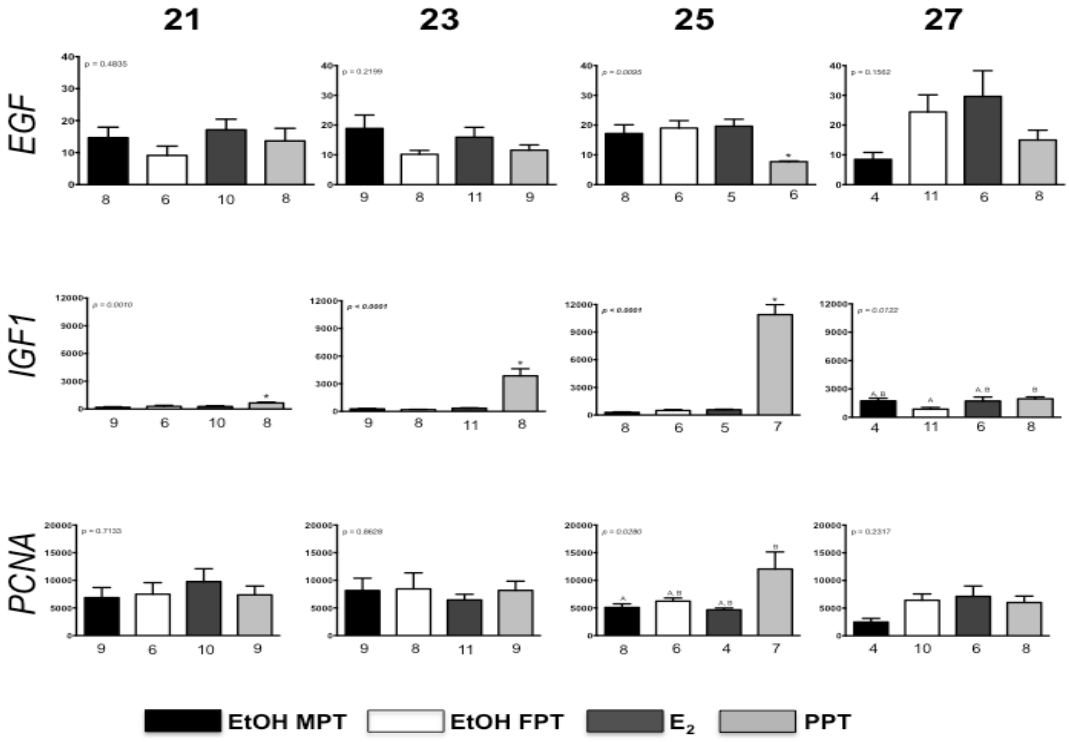


Fig. 3.8. *Effects of PPT treatment on growth factor expression in Müllerian duct at four developmental stages.* Relative expression of target gene normalized to calculated normalization factor. Error bars indicate mean standard error. Genes indicated to left of graphs, from top to bottom: *EGF*, *IGF1*, *PCNA*. Stage of collection above, from left to right: 21, 23, 25, 27. Bar color indicates treatment: black = 0.5 µl/g EW EtOH, MPT; white = 0.5 µl/g EW EtOH, FPT; dark gray = 0.5 µg/g EW E_2; light gray = 5 µg/g EW PPT. *p*-values via one-way ANOVA indicated above graphs. Sample size indicated below bars. Letters above bars indicate differences among treatment groups per Tukey's multiple comparison post hoc test.

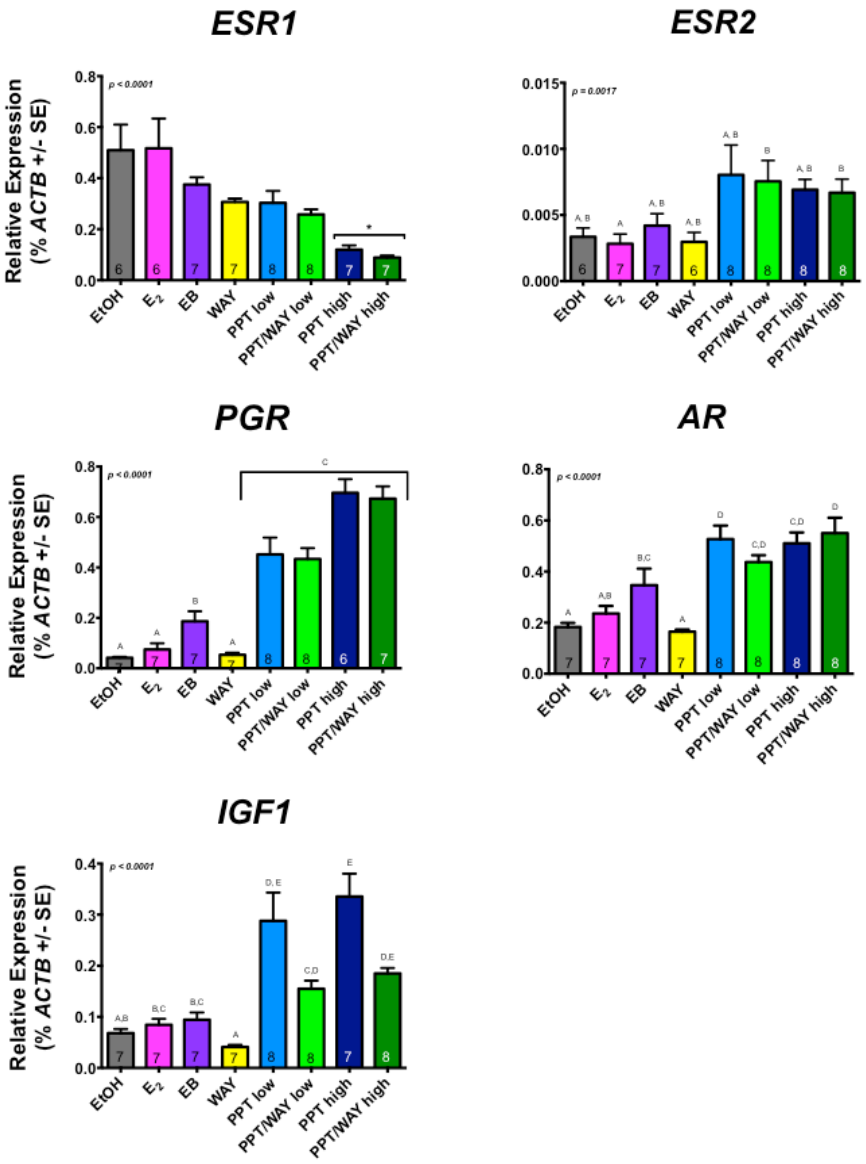


Fig. 3.9. *Effects of combinatorial treatments of PPT and WAY on expression of genes of interest in stage 27 Müllerian duct*

Expression values reported as relative expression normalized to *ACTB*. Error bars indicate mean standard error. Treatment indicated below bars. *p*-values via one-way ANOVA indicated above graphs. Sample size indicated below bars. Letters above bars indicate differences among treatment groups per Tukey's multiple comparison post hoc test.

CHAPTER 4:

The influence of background contaminant exposure on embryonic Müllerian duct response to *in ovo* stimulation with estrogen and ERα selective agonist PPT

4.1. Introduction

The American alligator has become a recognized wildlife model for studying the effects of developmental exposure to environmental endocrine disrupting contaminants in large part due to more than two decades of research on a population living in Lake Apopka, FL, a site of known heavy contamination with the pesticide DDT. The area around Lake Apopka has been used heavily for water-intensive agriculture known as muck farming, and DDT was used extensively on these fields until its ban in 1972 (Conrow et al., 2011). In 1980 there was a major spill of DDT into the lake from a nearby pesticide manufacturing plant (United States Environmental Protection Agency, 1994). In 1981, a significant decline in the population of juvenile alligators in Lake Apopka was observed, along with significantly depressed egg viability rates and hatching success compared with other Florida lakes (Woodward et al., 1993; Woodward et al., 2011).

Over the years, measurements of DDT and its metabolites, especially *p,p'*–DDE, have been higher in alligator eggs and tissues from Lake Apopka compared with other Florida lakes (Heinz et al., 1991; Rauschenberger et al., 2004; Woodward et al., 2011). DDE bioaccumulates in adipose tissue and thus levels remain consistent in populations over long time periods, and can increase in individuals chronically exposed over a long lifespan (Turusov et al., 2002). These types of adipose-accumulated contaminants can be transferred directly from mother to offspring when lipids are mobilized to produce nutritive breastmilk or yolk (reviewed in Hamlin and Guillette, 2011). This type of maternal transfer occurs in Apopka alligators, as DDT, DDE and other contaminants have been detected in both egg yolk and in maternal tissue (Rauschenberger et al., 2004; Rauschenberger et al., 2007). DDE at levels detected in yolk from Lake Apopka eggs has been shown to interact with alligator oviductal protein extract in competitive binding assays with estrogen or progesterone (Vonier et al., 1996).

Many anomalies have been documented in Apopka alligators compared with alligators from reference sites with lower measured contaminant levels. For most of these studies, Lake Woodruff National Wildlife Refuge has served as the reference site, as it has historically had fewer anthropogenic influences than Lake Apopka, as well as measurably lower levels of contaminants, and the alligator population has had much greater reproductive success (Milnes and Guillette, 2008). Egg viability rates and hatching success have remained consistently lower for Apopka than Woodruff (Guillette et al., 2000; Milnes and Guillette, 2008) and in studies where eggs were hatched out and maintained under identical laboratory conditions, Apopka alligators had higher post-hatching mortality than Woodruff alligators (Milnes et al., 2008). There have been several major morphological and physiological differences documented in alligators between these sites. Juvenile alligators in Lake Apopka had significantly decreased phallus size with lower plasma testosterone compared to Lake Woodruff (Guillette et al., 1996). Six-month-old alligators hatched from eggs collected at Lake Apopka and raised under controlled laboratory conditions exhibited disrupted gonadal morphology, most notably, multi-oocytic ovarian follicles reminiscent of those seen in mice exposed *in utero* to DES (Guillette et al., 1994; Guillette and Moore, 2006).

Molecular signals were investigated to probe the potential mechanisms underlying these ovarian alterations in Apopka alligators. Gonadal *ESR2* expression was higher in field-captured juvenile alligators in Lake Woodruff than in Lake Apopka (Kohno et al., 2008) and also in 13-month-old alligators collected as eggs from the field sites and reared under controlled laboratory conditions (Moore et al., 2010b). Laboratory-reared juvenile alligators collected as eggs from Lake Woodruff exhibited sexually dimorphic expression patterns of important genes involved in steroidogenesis and members of the transforming growth factor β (TGF-β) superfamily that promote and regulate ovarian follicle development, while juveniles collected as eggs from Apopka and raised under identical laboratory conditions did not (Milnes et al., 2008; Moore et al., 2011). At hatch, neonates from

Lake Apopka had lower gonadal expression of these ovarian folliculogenesis-regulating TGF-β factors than neonates from Lake Woodruff (Moore et al., 2010a). As measured by expression levels of folliculogenesis-regulating factors and steroidogenic enzyme aromatase, Apopka neonates exhibited a decreased response to challenge with FSH compared with Woodruff neonates (Moore et al., 2010a), a difference which persisted five months post-hatch (Moore et al., 2012).

These differences in morphology and molecular signaling in gonads of alligators from Apopka suggested this developmental exposure could also induce alterations in FRT. There are no reported differences in FRT morphology between Apopka and Woodruff alligators, but there are noticeable differences in eggs collected from these sites. Over the years we have collected eggs and incubated them in our laboratory, we have anecdotally noted Apopka eggs are smaller in size and weight than Woodruff eggs, and we have also observed malformed eggs from Apopka (unpublished observations). This suggests there may be impairments in reproductively mature Apopka FRT structure or function that impact egg production.

DDT and its metabolites have been famously implicated in major reproductive tract disorders in birds, particularly the phenomenon of eggshell thinning. In multiple, primarily carnivorous species, including pelicans, bald eagles, cormorants and herring gulls, decreased hatching success and significantly thinner eggshells (>15% reduction) were positively associated with levels of DDE in eggs, and laboratory exposure experiments confirmed eggshell thinning in birds exposed to DDE (Cooke, 1973). The mechanism of DDE-induced eggshell thinning is still under investigation. Originally it was thought that DDE interfered with prostaglandin synthesis in the shell gland of the avian oviduct, thus inhibiting calcium transport (Lundholm, 1997). More recent studies have shown that embryonic exposure to DDE can directly disrupt the development of the avian FRT, resulting in altered shell gland function and eggshell thinning (Holm et al., 2006; Kamata et al., 2009). Thus, exposure to DDE during a critical developmental window

may induce adverse impacts observed in birds, and alligators from Lake Apopka may be similarly susceptible to disrupted FRT development due to DDE exposure.

We have established that the developing alligator FRT is receptive to estrogenic stimulation via ERα, and treatment with the ERα agonist PPT induces hypertrophy and cellular differentiation in the MD. *In vitro* receptor transactivation assays have shown that DDT and DDE are human ER agonists, with greater activity on hERα than hERβ (Kojima et al., 2003). DDT and DDE also activate alligator ERα in *in vitro* receptor transactivation assays, though their effects on ERβ have not been assessed (Rider et al., 2010). Thus, exposure to DDE and other EDCs in Apopka alligators could lead to disrupted FRT development via selective stimulation of ERα.

Our previous investigations of the effects of ER isoform selective agonists on alligator MD provide a unique tool for testing this hypothesis. We have characterized the MD response to selective ERα activation via *in ovo* PPT treatment in alligator embryos from the relatively pristine environment of Lake Woodruff. Here we investigate whether background exposure to EDCs alters this ERα-mediated MD response by treating alligator embryos collected from Lake Apopka with PPT. We directly compare the morphological, histological and transcriptional effects of *in ovo* PPT treatment on MD in alligator embryos from Lake Apopka and Lake Woodruff.

4.2. Materials and methods

4.2.1. Egg collection, incubation and treatment

All experiments in this study involving alligators were carried out under the protocol approved by the Institutional Animal Care and Use Committee at the Medical University of South Carolina, and all fieldwork was conducted under permits from the Florida Fish and Wildlife Conservation Commission and the U.S. Fish and Wildlife Service.

Alligator eggs were collected from Lake Woodruff National Wildlife Refuge (De Leon Springs, FL, USA) on 6/20/2013 and Lake Apopka (Lake and Orange Counties, FL,

USA) on 6/21/2013 under Florida Fish and Wildlife Conservation Commission permits. Eggs were transported to MUSC facilities (Charleston, SC, USA), weighed, candled, staged and incubated at 30°C as described in the preceding chapters.

At stage 19, eggs were randomly assigned to treatment groups: 0.5 µL/g egg EW absolute ethanol; 0.5 µg/g EW E_2 (Sigma-Aldrich, St. Louis, MO, USA); 0.5 µg/g EW or 5 µg/g EW PPT (Tocris Bioscience, Bristol, UK), prepared as described in previous chapters. Doses were based on the previous experiments, in which these doses of PPT produced significant differences in MD morphology, histology and gene expression as compared with the vehicle control and E_2 treatment. Treatments were administered by eggshell painting as previously described, and embryos were incubated at FPT for the duration of the experiment.

Using the methodology shown in chapter 2, embryos were sacrificed at stage 27 and left and right GAM-MD complexes were collected under a dissecting microscope, the right side preserved in RNA*later*® (Life Technologies, Grand Island, NY, USA) and the left side in 4% PFA. The fixed tissues were incubated overnight on a shaker at 4°C. The RNA*later*® -preserved tissues were then stored at -20°C. The PFA-fixed tissues were progressively dehydrated in methanol/PBST over a 24-hour period and stored in 100% methanol at -20°C.

4.2.2. Histology

PFA-fixed tissues were dehydrated through a series of isopropanol and xylene and paraffin infiltrated using a Leica ASP300 autoprocessor (Wetzlar, Germany), and embedded into paraffin using a Leica EG1150C embedder. Tissues were serially cross-sectioned or serially lateral-sectioned at 8 µm using a Leica manual rotary microtome, and stained with hemotoxylin and eosin using a Leica Autostainer XL with a Leica CV5030 coverslipper.

For each treatment group, five samples were examined in full, 2-3 as serial cross sections and 2-3 as serial lateral sections. As in previous chapters, the anterior-posterior axis of the GAM was utilized as a metric for assessing regionalization of the MD. Representative digital photomicrographs were taken of cross-sectioned samples with a ProgRes Speed XT core 5 camera (Jenoptik, Jena, Germany) of a region near the anterior end of the GAM, where both the gonad and adrenal gland are present.

4.2.3. RNA isolation, cDNA synthesis and quantitative PCR

The methods for RNA extraction, cDNA synthesis and QPCR are similar to previous chapters, with minor modifications. RNA*later*® -preserved MD tissues were manually separated from GAM complexes under a dissecting microscope and stored at -20°C until RNA extraction. Whole MDs (n=8 per treatment group) were homogenized for RNA extraction via the SV Total RNA Isolation System with DNase-I treatment (Promega, Fitchburg, WI, USA). RNA concentration was measured using a Nanodrop Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and quality was assessed via denaturing agarose gel electrophoresis. RNA was diluted to matching concentrations and reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems, Foster City, CA, USA) per manufacturer's protocol, using 10 µL diluted RNA in 20 µL total reaction mix, and the cDNA was diluted 1:5 in nuclease-free water.

QPCR was performed using a CFX96 real-time system (Bio-Rad, Hercules, CA, USA). Reactions were performed in triplicate using 0.01 U/µl AmpliTaq Gold (Applied Biosystems, Carlsbad, CA, USA) and a SYBR green reaction master mix consisting of 20mM Tris-HCl (pH 7.75), 50mM KCl, 3mM $MgCl_2$, 4% DMSO, 0.5x SYBR Green I (Thermo Fisher Scientific, Grand Island, NY, USA), 0.5% glycerol, 0.5% Tween 20, 0.2mM deoxynucleotide mix (Thermo Fisher Scientific), 0.2 µM of each primer mix (Eurofins Genomics, Huntsville, AL, USA), and 1/25 volume diluted cDNA.

The thermal cycler program consisted of an initial denaturation step (95°C, 5 min) followed by 45 cycles of denaturation (95°C, 15 s) and annealing and elongation at an optimized temperature (45 s – 1 min, depending on primer set). After the final cycle, a dissociation curve was generated for each reaction to assess the reaction specificity via an incremental heating step (+0.5°C/5 s).

Primer sequences, product size and optimal annealing temperatures are listed in Table 1 (pg. 28). Prior to analysis of unknown samples, the specificity of each primer pair was confirmed by product sequencing. Plasmid DNA standard curves of known copy number were generated with these sequenced products. Each 96-well plate of unknown samples included a standard curve, and all samples, including those of the non-template control and standard curve, were run in triplicate. Mean expression values of mRNA for each target gene were normalized to that of beta-actin (*ACTB*).

4.2.4. Statistical analysis

QPCR data were collected and analyzed using CFX Manager version 3.0 software (Bio-Rad). After determining that expression of *ACTB* was not significantly affected by treatment or site (Apopka vs. Woodruff) via two-way analysis of variance (ANOVA), expression values of all other genes of interest were normalized to *ACTB*. Data were log-transformed to achieve homogeneity of variance. Two-way ANOVA was utilized to calculate the effects of treatment and site on expression of genes of interest. Outlying values were removed by examination of a residual plot and a distribution plot of the transformed data. Significance was set at $p < 0.05$, and a Tukey's multiple comparison post-hoc test was used to detect differences among treatment groups.

All statistical analysis was performed using both JMP12 (SAS Institute, Inc., Cary, NC, USA) and Graphpad Prism 6 (Graphpad Software, Inc., LaJolla, CA, USA) software.

4.3. Results

4.3.1. Morphometric differences between eggs and embryos from Lake Woodruff and Lake Apopka are not influenced by treatment

In the clutches of eggs utilized in this experiment, fertility rate was not significantly different between the sites, with 96% fertility for Lake Woodruff and 94% for Lake Apopka. As embryos were sacrificed prior to hatching, hatch rate could not be determined, but there was no significant difference between sites in mortality over the course of the experiment between dosing at stage 19 and sacrifice at stage 27 (n = 1 for Apopka and n = 2 for Woodruff). When eggs were initially brought to the laboratory and candled for viability, they were weighed, and there was no difference in mean egg weights between the sites for the particular clutches utilized in this experiment.

At time of sacrifice, eggs and embryos were weighed, and there was a significant difference in egg mass between Woodruff and Apopka (Woodruff = 90.3 0.089g, Apopka = 80.9 0.084g, two-tailed $p < 0.0001$). There was no significant difference in embryo mass, however (Woodruff = 35.5 0.9 g, Apopka = 33.6 0.06 g, two-tailed t test $p = 0.089$).

4.3.2. PPT treatment induces similar changes in Müllerian duct morphology and histology in embryos from both Lake Woodruff and Lake Apopka

PPT treatment induced the same MD gross morphology in this experiment as previously described in chapter 2 (as shown in Fig. 2.1). Compared with MD of controls and E_2 treatment, the MD of stage 27 embryos was massively enlarged in embryos treated with 5 µg/g EW PPT, and somewhat enlarged in embryos treated with 0.5 µg/g EW PPT. Within treatment groups, there were no appreciable differences between Woodruff and Apopka embryos at stage 27. PPT-treated MDs were weighed prior to RNA extraction for the purposes of proper reagent calculation, and MD masses between Woodruff and Apopka samples was not significantly different (Woodruff = 51.38 7.84 g, Apopka = 35.75 4.02 g; two-tailed t-test $p = 0.098$).

The histological findings were also consistent with what was described in chapter 2. In the vehicle control samples and the E_2-treated samples, the stage 27 MD was a simple tube with a columnar epithelium surrounding the lumen and a fibroblastic mesenchymal stroma (Fig. 4.1 A-D). Treatment with 0.5 µg/g EW PPT induced enlargement of the MD that involved stromal hypertrophy, as described in ch. 2, in both Woodruff and Apopka samples (Fig. 4.1 E, F). At the highest dose, 5 µg/g EW, PPT treatment induced the phenotype of MD hypertrophy with precocious glandular differentiation detailed in previous chapters. There were no observable differences between Apopka and Woodruff embryos at stage 27 in this treatment group (Fig 4.1. G, H).

4.3.3. PPT treatment induces similar changes in expression of genes of interest in Müllerian duct in embryos from both Woodruff and Apopka

As in previous experiments, PPT treatment induced significant changes in expression of SHRs and *IGF1* compared with vehicle control and E_2 treatment (Fig. 4.2)

Treatment with 5 µg/g EW PPT reduced MD *ESR1* about fourfold in both Woodruff and Apopka samples compared with control and E_2-treated samples. *ESR1* was somewhat reduced in 0.5 µg/g EW PPT-treated Woodruff MD samples, but not in Apopka samples. *ESR2* was increased in 0.5 µg/g EW and 5 µg/g EW PPT-treated samples compared to controls, in both Woodruff and Apopka MDs. This increase was greater in samples from Apopka than in Woodruff, with a five-fold increase in *ESR2* expression in PPT-treated Apopka MDs compared to controls, and only a two-fold increase in PPT-treated Woodruff MDs compared to controls.

PPT treatment significantly increased expression of *PGR* compared to controls and E_2 treatment in both Woodruff and Apopka MDs. While *PGR* levels were similar in both 0.5 µg/g EW and 5 µg/g EW PPT-treated Woodruff samples, in Apopka samples, 5 µg/g EW PPT treatment induced significantly higher *PGR* expression than 0.5 µg/g EW PPT treatment.

For both *AR* and *IGF1*, expression was significantly higher in PPT-treated MDs than controls or E_2-treated MDs in both Woodruff and Apopka samples, with no significant differences between the sites.

4.4. Discussion

Overall, the MD response to PPT treatment in Apopka embryos was very similar to that of Woodruff embryos. There are no previously documented morphological or histological differences between MDs or immature oviducts from Apopka and Woodruff alligators, which is unsurprising in light of our findings documented in previous chapters. The developing alligator MD only exhibits morphological changes through *in ovo* treatment with extreme doses of estrogens or progestins (Austin, 1991; Lang and Andrews, 1994) or via selective activation of ERα as our experiments have shown. DDE and other contaminants present in Apopka egg yolk are unlikely to be as highly endocrine active. Indeed, DDT and its metabolites were shown to have low affinity for alligator oviductal protein extract in competitive binding assays with estrogen and progesterone, although mixtures of contaminants found in Apopka eggs had a greater than additive effect (Vonier et al., 1996). *In vitro* receptor transactivation assays also showed DDT and DDE were weakly activating of alligator ERα (Rider et al., 2010). Experimental treatment of alligator embryos from Lake Woodruff with 100 ppb DDE increased female:male sex ratio at the intermediate incubation temperature, but did not alter MD morphology or MD epithelial cell height (Milnes et al., 2005).

No major oviductal differences have been reported in adult Apopka alligators, but differences in egg mass, morphology, viability and hatching success have all been documented. While eggshell thinning has been highly correlated with DDE exposure in birds, no studies support DDE-induced eggshell thinning in alligators. In 1985, there was no significant difference found between shell thickness of eggs from Lake Apopka and a reference site, and there was even a slight positive correlation between DDE residues in

eggs and eggshell thickness (Heinz et al., 1991). A multi-year study of eggshells collected after laboratory hatch-out found Apopka eggs actually had thicker shells than Woodruff eggs (Bryan, 2005).

While the levels of DDE measured in alligator eggs in Lake Apopka are consistently higher than those measured at other sites, they are lower than levels documented in bird populations that were significantly impacted by DDE-induced eggshell thinning. Between 1984 and 2002, DDE levels in Apopka alligator eggs were measured several times and average levels ranged from 3.5 - 5.8 ppm (Heinz et al., 1991; Rauschenberger et al., 2007; Woodward et al., 2011). A study of bald eagles suffering a serious DDT-associated population decline in Ontario reported levels of DDE in eggs as high as 94 ppm prior to the ban of DDT, and by1981 levels had dropped to a mean of 29 ppm and both hatching rate and eggshell thickness had improved significantly (Grier, 1982). However, avian FRT anomalies have been documented at lower levels of DDE exposure as well. In brown pelicans, 4 - 5 ppm DDE in eggs was correlated with 15% eggshell thinning, and reproductive impairment occurred above 2.5 ppm (Blus et al., 1974).

More recent laboratory studies have directly correlated *in ovo* exposure to DDT with changes in reproductive system patterning that lead to eggshell thinning in chickens (Holm et al., 2006) and Japanese quail (Kamata et al., 2009). This impairment in oviductal function is accompanied by obvious structural changes including foreshortening of the left oviduct and retention of all or part of the right oviduct, which normally completely regresses in these birds (Holm et al., 2006; Kamata et al., 2009). These types of oviductal anomalies are also seen in birds following *in ovo* exposure to other estrogens and estrogenic contaminants such as DES, ethinyl estradiol, EB and bisphenol A (Berg et al., 1999; Brunstrom et al., 2009; Halldin, 2005; Rissman et al., 1984).

Taken together, it is appears that alligator MD differs from avian MD in responsiveness to estrogenic stimulation. It takes a large, imbalanced ERα stimulation to induce observable morphological changes in alligator MD similar to those observed in avian

studies utilizing much lesser estrogenic stimuli. However, the alligator MD does show estrogenic treatment-induced alterations on a more subtle molecular level. In a study of 5-month-old laboratory-reared alligators, FSH challenge elicited an increase in transcript levels of *PGR* and *AR* in oviducts from animals collected as eggs from Lake Woodruff and not in those from Lake Apopka. In this study, we also found subtle differences in *SHR* expression between Woodruff and Apopka alligators, with Apopka alligators exhibiting larger PPT treatment-induced increases in *ESR2* and *PGR*.

In previous chapters we have posited that *ESR2* and *PGR* expression is altered by PPT treatment as a feedback response, as negative regulators of *ESR1* signaling. The differences in PPT-induced expression changes of these genes in Apopka embryos compared with Woodruff embryos suggests there may be alterations in steroid hormone regulatory responses in Apopka alligators.

In conclusion, developmental exposure to endocrine disrupting contaminants at ecologically relevant doses, as represented by yolk contaminants in embryos from Lake Apopka, does not have any observable impacts on the developing alligator MD. However, this background exposure can result in altered molecular signaling responses to additional endocrine stimulus, such as the treatment regime in this experiment. The repercussions of this altered response on FRT function and, by extension, the overall reproductive health of alligators, is not known and requires further investigation.

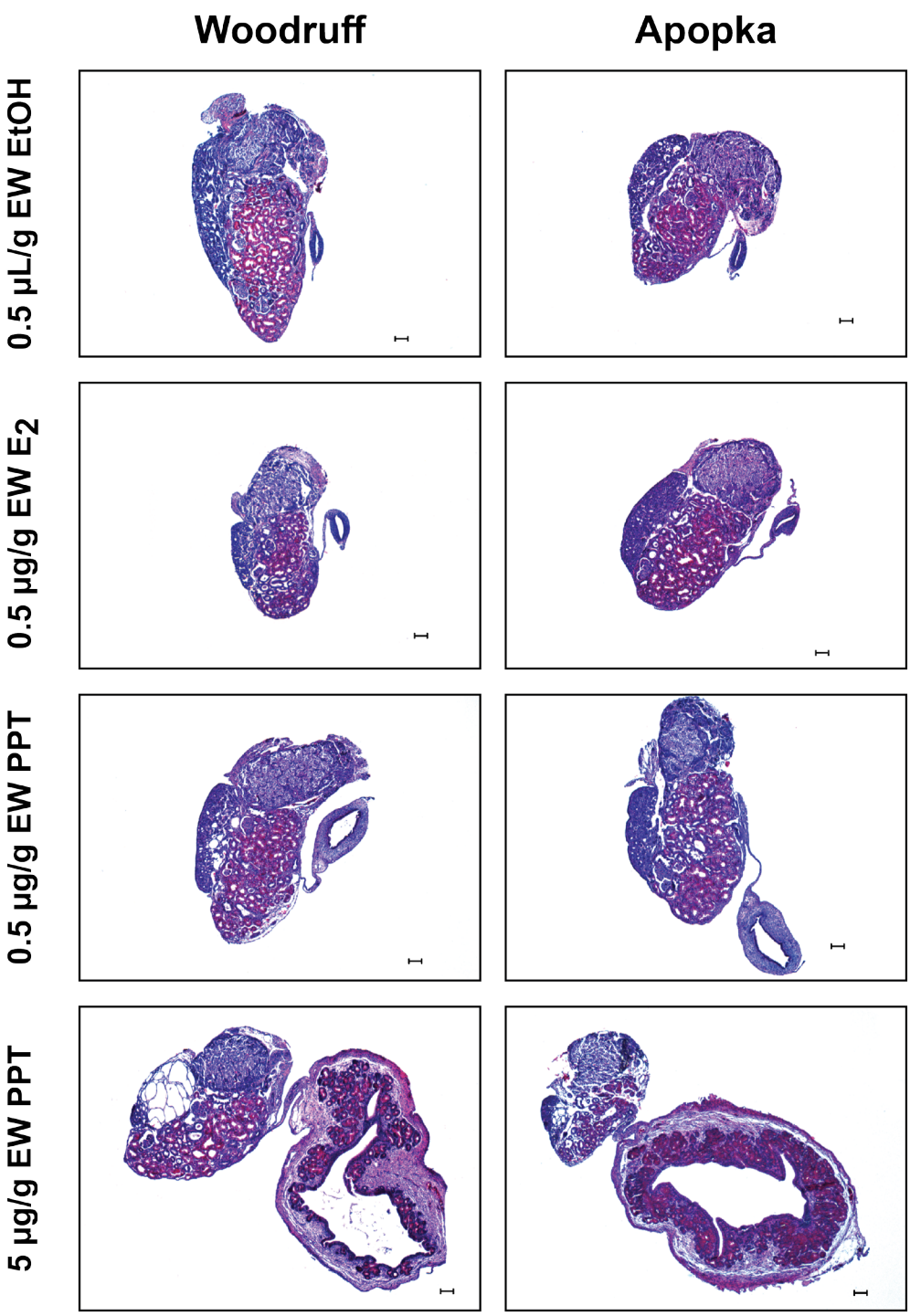


Fig. 4.1. *Effects of PPT treatment on histological characteristics of stage 27 Müllerian duct in alligators from a reference site vs. those from a site of known contamination.*
Representative cross sectional photomicrographs of FPT stage 27 alligator GAM and MD from Lake Woodruff (left) and Lake Apopka (right). Treatments indicated to the left of images, from top to bottom: 0.5 µL/g EW EtOH, 0.5 µg/g EW E_2, 0.5 µg/g EW PPT and 5 µg/g EW PPT. Stained with H&E. Scale bars = 100 µm

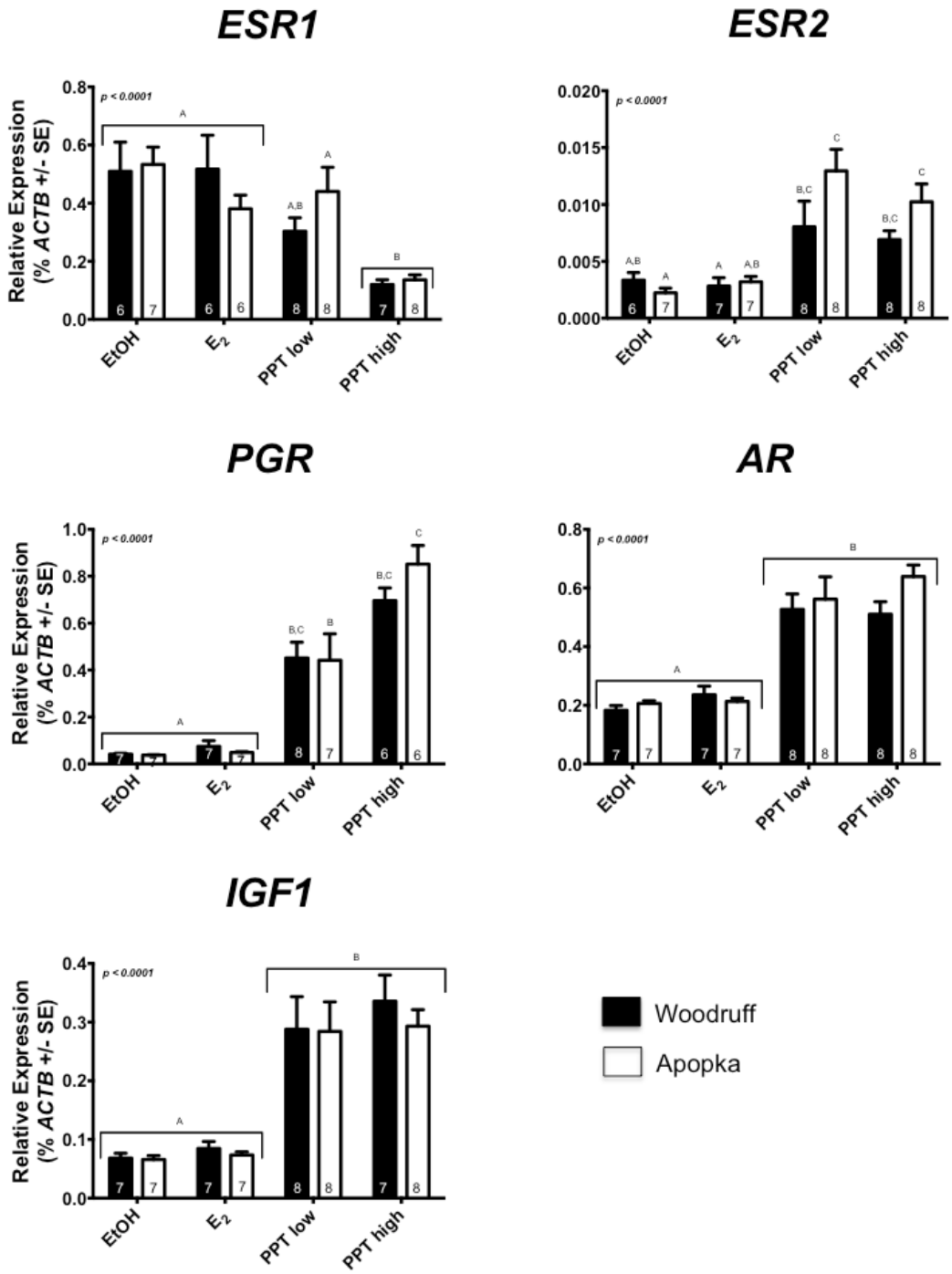


Fig. 4.2 *Effects of PPT treatment on expression of genes of interest in stage 27 Müllerian duct from alligators from a reference site vs. those from a site of known contamination*. Expression values reported as relative expression normalized to *ACTB*. Error bars indicate mean standard error. Treatment indicated below bars. Black bars = Lake Woodruff samples, white bars = Lake Apopka samples. *p*-values via two-way ANOVA indicated above graphs. Sample size indicated below bars. Letters above bars indicate differences among treatment groups per Tukey's multiple comparison post hoc test.

CHAPTER 5:

General discussion

5.1. Summary

The overarching goal of this dissertation research was to investigate the development of the female reproductive tract of the American alligator in the context of endocrine disruption. The endocrine disruption hypothesis has become an established paradigm guiding investigations that are critical for the health of humans and all other life on the planet, which makes it by necessity broad and transdisciplinary. The foundations of this field are "basic science" investigations, however; and when I entered graduate school and expressed my lofty aims to my mentor, Dr. Louis Guillette, I quickly learned one of his most often-repeated maxims, "You can't understand 'abnormal' until you understand 'normal.'"

In the case of alligator FRT development, "normal" was far from established. Studies on alligator reproductive physiology examined mature FRT and positioned the alligator FRT as closer, from an evolutionary standpoint, to birds than to other reptiles, though it displayed characteristics of both (Palmer and Guillette, 1992). There is a dearth of studies on the developmental differentiation of alligator FRT, ostensibly due to the difficulty of studying a slow-maturing, long-lived, large predator.

The embryonic alligator MD is a simple, uniform tube. The only documented regional difference in normal female alligator MD was discussed in ch. 2: the MD region at the posterior terminus of the adrenal gland of the GAM displays a thickened luminal epithelium. This characteristic is only present for a very short portion of the MD, and its significance is unknown, but it was consistently seen in all FPT control MD, suggesting that it is a normal characteristic of developing alligator FRT (Doheny et al., 2016). The age at which the alligator FRT normally becomes regionally differentiated under natural conditions is unknown. Differentiation of the oviduct has not been reported in any laboratory studies involving untreated female alligators reared from hatching until as old as 1.5

years of age (Forbes, 1938b). In my own experience necropsying female alligators reared for about 6 months post-hatch in the laboratory, until they had attained one kg body mass, the oviducts were simple, thin tubes for nearly their entire length, with some sinuous curving at the anterior end. Histologically, no major differences were seen in serial cross section or in lateral sectioning of coiled tissues; the oviducts were similar to the embryonic MD, with a central lumen surrounded by a simple columnar epithelium and a fibroblastic stroma.

The age at which alligators are reproductively mature is variable, ranging from 10-18 years, depending on temperature and nutrient availability (Lance, 2003). The more important determinant of maturity is body size, and the most reliable, standardized morphometric used in studies of alligator populations is the length from the tip of the snout to the vent (SVL). Alligators attain reproductive maturity at a SVL of 90 cm (Wilkinson and Rhodes, 1997). From the aforementioned unpublished study we conducted in which alligators were hatched out and reared in the lab until they reached one kg in mass, the average SVL at hatch was 12.3 ± 0.4 cm, and the SVL at sacrifice was 33.5 ± 0.9 cm. There is some evidence to suggest that alligators enter peripubescence above a threshold size of approximately 38 cm SVL, as wild juvenile alligators begin to show pronounced seasonal variation in circulating sex hormones (Rooney et al., 2004). Thus, differentiation of the alligator oviduct normally occurs after it becomes prohibitive to maintain alligators in the laboratory due to their size, and likely involves hormone signaling during a pubertal phase.

While still pondering the best methods for studying alligator FRT development given this major limitation, as part of a different study aimed at investigating gonadal sex determination we unexpectedly induced a hypertrophied MD phenotype heretofore never seen in our research group. This experimentally-induced phenotype, while not a direct reflection of "normal" FRT development, nevertheless provided a model for investigating how the embryonic MD responds to estrogenic stimulation, and the implications of this response for the process of normal FRT differentiation.

Over the course of this investigation, I established the basic characteristics of the MD phenotype induced via treatment with the selective ERα agonist PPT. The massive MD enlargement easily observed with the naked eye during necropsy was examined at a histological level, initially to determine whether this enlargement was due to hypertrophy or hyperplasia. I discovered that while there was hypertrophy of stromal cells, the more striking characteristic was differentiation of luminal epithelial cells into glandular structures similar to the process of adenogenesis in the mammalian and avian FRTs. Numerous studies have detailed induction of glandular formation in avian and mammalian FRT by developmental exposure to endogenous and exogenous estrogens and estrogenic contaminants (reviewed in Dougherty and Sanders, 2005; Kurita, 2011). My discovery of glandulogenesis in embryonic alligator MD is thus not unique if considered in the context of the FRT development in amniotes, but the intriguing aspect of it is that only treatment with a pharmacological selective agonist of ERα induced this glandulogenesis. As detailed in the introduction, previous studies involving treatment with E_2, synthetic estrogens, or estrogenic compounds, did not induce this response in alligators (Austin, 1989, 1991; Forbes, 1938b). Both the developing mammalian and avian FRTs seem much more labile to estrogen signaling than the alligator, as major FRT structural changes have been reported in birds and mammals following embryonic exposure to estrogens (reviewed in Dougherty and Sanders, 2005; Iguchi, 2000; Kurita, 2011).

The novelty of the PPT-induced phenotype in alligator MD could well be due to the fact that alligators are simply not that well-studied; if the alligator was a more popular and accessible model, more rigorous testing of endocrine active compounds during alligator development might have resulted in other incidences of this phenotype. Alternatively, the factors determining alligator FRT differentiation could be quite different from those guiding differentiation in more well-studied models with genetic, rather than environmental, sex determination. In mammals, where *SRY* on the Y chromosome guides male sex determination, the XX female is considered the “default” state, with ovarian differentia-

tion occurring in the absence of *SRY*, and FRT differentiation occurs in a similar default fashion, in the absence of male-determining endocrine factors (Gilbert, 2000; vom Saal et al., 1992). In birds, the ZW female only receives a "half-dose" of the Z-linked male-determining factor DMRT1, and thus ovarian differentiation also occurs in the absence of the male signal (Smith et al., 2009). Designating FRT differentiation as a the default system in birds is a more complicated picture than in mammals, however, as AMH is produced in both males and females, and ovarian estrogens are necessary for protecting the FRT against AMH-induced degeneration (Hamilton, 1961). This begs the question: in alligators and other animals with temperature dependent sex determination, is there a "default" system? What factors actually drive reproductive tract differentiation?

Interestingly, studies have demonstrated that estrogens administered prior to the thermosensitive window of sex determination can induce ovarian development in alligators incubated at MPT, indicating that estrogens can override the male-determining thermal signal, while thus far no androgenic treatment has induced testis development at FPT (Kohno and Guillette, 2013; Lance and Bogart, 1992). This may indicate that female is the "default" state in alligators, i.e., in the absence of a male-determining signal, female differentiation occurs. This idea is supported by the fact that there is a much larger range of temperatures that bias female differentiation in the alligator and a larger window of time in which these temperatures can bias toward female sex determination (Kohno and Guillette, 2013; McCoy et al., 2015). Once gonadal differentiation has occurred, testis-produced AMH leads to MD degeneration, which is completed prior to or not long after hatch out, whereas in the female, the MD is maintained but remains undifferentiated until much later. Experimental evidence suggests that estrogen can be protective against MD regression in alligators, but is not necessary in normal female development. When female neonates were ovariectomized and given a graft of testicular tissue, the MDs regressed, but MD regression did not occur in ovariectomized neonates given a graft of muscular tissue, nor in ovariectomized, testis-grafted females implanted with a slow-re-

lease pellet of E_2 (Austin, 1989). Thus, perhaps the best descriptor for the developing alligator FRT is not "default" but rather "passive," as it can be responsive to exogenous estrogen stimulation, particularly via ERα, but does not need estrogenic signals during embryonic development. The immature FRT remains undifferentiated until it receives the appropriate signal driving regionalized cytodifferentiation at a much later developmental time point.

5.2. Future directions

A role for estrogen signaling in this later regionalized differentiation of the alligator FRT is strongly suggested by experiments described in the previous chapters characterizing the regionalized cytodifferentiation of the MD induced by *in ovo* PPT treatment. Across all of the experiments described, stage 27 alligator MD treated at stage 19 with 5 μg/g EW PPT had a greater density of glandular structures in the anterior portion of the MD, decreasing posteriorly. This could be due to the treatment operating on an anterior-posterior gradient, and having a stronger effect on the anterior regions, or it could be due to underlying factors guiding regional differentiation of the FRT. The adult alligator FRT is highly regionalized, and the anterior tube similarly displays the greatest density of glands (Palmer and Guillette, 1992). Many genetic factors have been identified that are involved in regional differentiation of the mammalian FRT (Kobayashi and Behringer, 2003) and it would be worthwhile to investigate similar factors in alligator FRT differentiation.

In investigations of these regulatory factors involved in FRT differentiation, a target of particular interest are the Homebox (Hox) genes, due to their ubiquity in axis-determining patterns in embryos. As mentioned in chapter one, regional expression of the Hoxa 5' genes correlate with the regional differentiation of the FRT in mammals (Ma et al., 1998; Taylor, 2000), and experimental evidence suggests that estrogens regulate regional expression of Hoxa 5' genes in the developing FRT (Akbas et al., 2004). Devel-

opmental exposure to the synthetic estrogen diethylstilbestrol in mice results in a posterior shift of Hoxa9, Hoxa10 and Hoxa11 gene expression, and reproductive tract abnormalities indicative of a posterior shift of specialized structures (Block et al., 2000; Ma et al., 1998). Thus, future investigation into the effects of treatment with E_2 and estrogen receptor-selective agonists on Hox gene expression in specific regions of the alligator MD could be valuable in better understanding regional patterning of FRT differentiation in alligators and other vertebrates.

Further, a greater understanding of the molecular mechanisms underlying the development of the alligator oviduct could inform general understanding of the evolution of viviparity. Hoxa10 has been shown to have an important role in successful implantation and maintenance of pregnancy in mammals, which has led to investigations into its role in structural adaptations associated with the transition from oviparity to viviparity. Hoxa10 knockdown in cycling mouse uterus prior to implantation results in decreased formation of pinopods, projections from nonciliated endometrial epithelium that are associated with implantation success (Bagot et al., 2001). Pinopods have been documented in viviparous, but not oviparous reptiles, and different isoforms of Hoxa10-like proteins were found in the adult oviducts of an oviparous lizard (*Lampropholis guichenoti*) and a viviparous lizard (*Eulamprus tympanum*) (Thomson, 2006; Thomson et al., 2005). Under this hypothesis of uterine Hoxa10 expression being associated with viviparity, considering the evolutionary Archosaurian lineage of alligators and other crocodilians, detecting Hoxa10 gene expression in the alligator FRT would be an interesting finding.

I proposed to investigate regionalized Hox gene expression in the alligator MD using *in situ* hybridization when I was awarded a fellowship through the National Science Foundation and the Japan Society for the Promotion of Science to work in the laboratory of Dr. Taisen Iguchi. By utilizing a sample set from my experiments involving treatment with E_2 and PPT, I planned to determine whether Hox genes were expressed in alligator MD at all, using the control samples, and then whether normal expression patterns were

altered by estrogenic treatment. Unfortunately, I was not issued the necessary CITES permit to export alligator tissues to Japan in time, and without the expertise of my Japanese colleagues I was unable to complete this experiment. I believe this remains an intriguing and potentially highly informative area for further research into the development of the alligator FRT.

5.3. Conclusions

As an intial foray into a complex developmental process in a non-model species from a complex environment, this study had its limitations, but still provides some valuable insights into potential regulatory mechanisms involved in alligator FRT differentiation. The discovery that the alligator MD can be influenced to precocious glandulogenesis by developmental exposure to PPT is significant for its novelty in the alligator, particularly because it demonstrates a bias of signaling through ERα in the differentiation of the FRT. The experiments described in this dissertation support the hypothesis that stimulation of ERα via a selective agonist, without effective ERβ stimulation to play a regulatory role, induces proliferation and differentiation in the MD. While these experiments did not clearly define the role of signaling through ERβ in regulating the proliferative and differentiative effects of ERα signaling on alligator FRT differentiation, they provided supportive evidence that the effects of PPT are most likely due to its selective ERα activation rather than non-target effects of this compound.

As with any scientific investigation, this dissertation produced more questions than it answered, particularly by illuminating the gaps in the general understanding of the normal process of FRT differentiation in alligators, and even all other vertebrates evaluated for comparative purposes. It seems that the convention of considering reproductive tract differentiation to be “default,” simply an accessory to gonadal differentiation, is short-sighted. There are important signaling factors known to be involved in regulating regional patterning of the FRT, and there is strong evidence that these factors are regu-

lated by steroid hormone signaling. The role of endocrine signaling in the developmental differentiation of the MD to the regionally specialized structures of the mature FRT is clouded by the role of endocrine signaling in the reproductively mature FRT itself, as it must be remarkably plastic and hormone-responsive in order to dramatically increase in size and provide the necessary components for fertilized eggs to develop into healthy offspring. This means that estrogen signaling can play a different role in the embryonic development of the FRT than in the later remodeling of the reproductively active FRT, which makes it difficult to draw conclusions from experiments involving treatment of developing embryos with exogenous estrogens.

All in all, the female reproductive tract is evolutionarily and developmentally remarkable: it originates as a very simple tube composed of two cell types, and under the influence of a proper signaling environment, transforms into a structure of specialized regional functionality that is capable of packaging offspring into a protected, nutrient-filled environment or directly connecting offspring to the mother's body for developmental nurturing. In short, a properly functioning FRT is crucial for producing healthy offspring, and proper functioning of the FRT is reliant on proper developmental patterning. The modern world has put this process in jeopardy, with the prevalence and ubiquity of EDCs released into the environment. If we are to protect the future of life on this planet, our only recourse is to seek greater understanding of the fundamental "normal" processes guiding the development of the FRT, in order to protect these processes against "abnormal."

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Biographical Sketch

Brenna Mary Doheny was born March 16, 1981, in Minneapolis, Minnesota, but at age 7 her family relocated to the sunny beaches of Northwest Florida. Brenna spent a good part of her childhood snorkeling in the bayou in her backyard, kindling a lifelong interest in marine biology. Her love of learning led her to strong academic achievement in the International Baccalaureate Program at her high school, and she graduated *summa cum laude*. Brenna postponed college in favor of accepting a Congress-Bundestag Youth Exchange Program scholarship to spend a year as an exchange student at the Gymnasium Neue Oberschule in Braunschweig, Germany, where she cultivated fluency in the German language and a love of traveling and experiencing new cultures.

Upon returning to the U.S., Brenna began studying biology at the University of West Florida, and in the summer following her freshman year, she began an internship with the U.S. EPA through their Science Training in Ecology Program, under the mentorship of William P. Davis. This internship was her introduction to the field of environmental endocrine disruption, which quickly became her greatest scientific passion.

Funding cuts led to the termination of the STEP program, however, and seeing a lack of other interesting research opportunities at UWF, Brenna began attending Oregon State University through the National Student Exchange program. She spent an academic quarter at OSU's Hatfield Marine Science Center and interned with NOAA's National Marine Fisheries Service, studying pathogen transmission in juvenile salmon. She arranged a summer research internship with Jean Joss at Macquarie University in Sydney, Australia, investigating the evolution of tetrapod limb development with Australian lungfish.

Brenna transferred to OSU to finish her B.S. in biology, and added a B.A. in German in order to maintain her language skills. She pursued a side interest in science journalism as a reporter and editor for the student-run daily campus newspaper, and won a regional award for her writing. She also pursued her interest in public outreach in

environmental health and sustainability, and started a community research and advocacy group promoting the use of biodiesel. This group was awarded a grant from the U.S. EPA to present their work at an event on the National Mall in Washington, D.C. As part of their presentation, Brenna and two fellow group members drove all the way across the country to this event in her 1978 diesel engine Mercedes 300D, fueled by biodiesel.

After graduating *magna cum laude* from OSU, Brenna followed her sense of "Wanderlust" to Austria, where she spent two years as an English teaching assistant through the Fulbright program. By the end of this time spent honing her language and teaching skills while exploring Europe, it was time to return to her passion for endocrine disruption research. She entered the PhD program in Marine Biomedicine and Environmental Sciences at the Medical University of South Carolina under the mentorship of Louis J. Guillette, Jr., one of her personal heroes.

While pursuing her dissertation research on the developing female reproductive tract in the American alligator, Brenna served as a teaching assistant at the Citadel through the Graduate Assistance in Areas of National Need grant program. She also mentored undergraduate researchers in the laboratory and helped to coordinate the MBES Summer Undergraduate Research Program. Brenna led the MBES Student Association in outreach events promoting education in environmental health issues, for which she received funding through the SC SEA Grant Consortium.

In 2014, Brenna was awarded a fellowship through the NSF East Asia Pacific Summer Institutes and the Japan Society for the Promotion of Science Summer Program to spend 10 weeks in Japan, working in the laboratory of Taisen Iguchi, a longtime research collaborator with Louis Guillette.

After being awarded her PhD from MUSC, Brenna will continue pursuing her passion for environmental health research and sharing this research through public outreach and science education. She plans to take advantage of any opportunities to travel and experience new cultures along the way.